Development of functional protein microencapsulates by combining of milk protein ingredients aqueous solutions with blackcurrant concentrate, and encapsulating via spray-drying and freeze-drying, and the applications of microencapsulates in food products

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A thesis

submitted in partial fulfilment

of the requirements for the Degree of

Doctor of Philosophy

at

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by

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Abbreviation

ALA	α-lactalbumin
BSA	Bovine serum albumin
BLG	β-lactoglobulin
C3G	Cyanidin 3-O-glucoside
C3R	Cyanidin 3-O-rutinoside
D3R	Delphinidin 3-O-rutinoside
D3G	Delphinidin 3-O-glucoside
DPPH	2,2-diphenyl-1-picrylhydrazyl
EE	Encapsulation efficacy
FWC	Freeze-drying + Whey protein isolate + Imitation blackcurrant juice (Control)
FWB	Freeze-drying + Whey protein isolate + Blackcurrant concentrate
FNaC	Freeze-drying + Sodium caseinate + Imitation blackcurrant juice (Control)
FNaB	Freeze-drying + Sodium caseinate + Blackcurrant concentrate
FRAP	Ferric reducing antioxidant power assay
HPLC	High performance liquid chromatography
IBJ	Imitation Blackcurrant juice
LA	Lactoferrin
NaCas	Sodium Caseinate
OHC	Oil holding capacity
SAC	Surface anthocyanin content
SEM	Scanning electron microscopy
SWC	Spray-drying + Whey protein isolate + Imitation blackcurrant juice (Control)
SWB	Spray-drying + Whey protein isolate + Blackcurrant concentrate
SNaC	Spray-drying + Sodium caseinate + Imitation blackcurrant juice (Control)
SNaB	Spray-drying + Sodium caseinate + Blackcurrant concentrate
TAC	Total anthocyanins content
WPC	Whey protein concentrate
WPI	Whey protein isolate
WPH	Whey protein hydrolysate
WPI	Whey Protein Isolate
WHC	Water holding capacity

Abstract of a thesis submitted in partial fulfilment of the

requirements for the Degree of Doctor of Philosophy.

Novel functional protein ingredients and their applications

by

Gang Wu

Whey protein and sodium caseinate play a vital role in many food systems and exhibit multifunctional properties in food processing which enhance physical and nutritional values. The main functional properties, such as water holding capacity, oil holding capacity, foaming, and emulsification, are important charicteristics in food development and production. Among all of the commercially avaliable protein ingredients, both whey protein isolate and sodium caseinate, which are sourced from bovine milk, are widely used as protein ingredients for industial production, such as yogurt and baking products. These milk protein ingredients are nutritious, used for the building and repairing of tissues, fighting against infections, and providing energy for metabolism. Whey protein is obtained from cheese processing by-product-whey. Broadening whey protein's application is beneficial for dairy industry sustainability and the valorisation of whey protein as a functional ingredient. The functional properties of these proteins are based on their biomolecular structures, which can be modified by several compositional and processing factors.

Blackcurrant is abundant in health beneficial phytochemicals, such as vitamin C, polyphenols, and anthocyanins. Due to the intense sour taste, fresh blackcurrant fruits are always processed into juice concentrate for further food application. The bioactive compounds in blackcurrant juice concentrate are unstable in a free form, especially when they undergo

further food processing conditions, and also in the human digestive systems. This can contribute to a low bioaccessibility and bioavailability for its health benefit components, especially anthocyanins. The development of further applications for blackcurrant juice concentrate in a food system is of vital importance to aid the sustainable development of the blackcurrant crop industry, and bringing the goodness of this fruit to a wider population.

Combinations of common protein ingredients with fruits and vegetables concentrates or extracts are regarded as an effective ingredient to create novel protein products, to explore the application of concentrates or extracts from fruits and vegetables. Many studies have focused on the modification of protein structures, and the alteration of protein functionalities under controlled conditions. However, few studies have focused on the development of novel protein ingredients in a food-compatible manner, and their further incorporation in real food system.

Whey protein isolate and sodium caseinate have been commonly used as delivery systems for functional components due to their wide availability, nutritional values, and protective effects on sensitive bioactive components. In this study, blackcurrant juice concentrate was encapsulated via spray-drying and freeze-drying strategies by utilising whey protein isolate or sodium caseinate as the wall material without applying any food additives, obtaining four different kinds of novel protein ingredients (SWB, FWB, SNaB, FNaB) with different physicochemical, functional, and nutritional characteristics. The nutritional, physicochemical and functional properties of these ingredients were determined and defined. The results indicated that these ingredients could be used as natural colourants, protein enhancers, antioxidants, replacement of carbohydrate. The novel functional protein powders were then further incorporated into real food matrix-cookie at various levels (0%, 5%, 10%, 15%) to develop functional snack (cookie). Dough and cookie physicochemical properties, and cookie nutritional qualities were determined. The protein ingredients gave cookie products with

different colour and texture, increased antioxidant power, and most importantly generated hypoglyceamic effects. Collectively, these results illustrate that blackcurrant concentrate health benefits can be delivered to food systems via milk protein ingredients-based encapsulates.

Keywords: whey protein isolate, sodium caseinate, blackcurrant concentrate, polyphenol, anthocyanin, interaction, encapsulation, natural colourant, antioxidant, anti-cancer, glycaemic response, functional ingredient, functional properties, functional cookies, spray-drying, freeze-drying

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Chapter 1

Introduction and thesis outline

1.1 Introduction

Various milk protein ingredients have been used as carrier agents for dietary phenolics due to their molecular interactions and protective effects on sensitive phenolics. Dietary polyphenols are recognised as nutraceuticals which can act to prevent the onset of chronic diseases (Castro-Acosta *et al.*, 2017; Cory, Passarelli, Szeto, Tamez, & Mattei, 2018; Foegeding, Plundrich, Schneider, Campbell, & Lila, 2017; Tressera-Rimbau, Arranz, Eder, & Vallverdú-Queralt, 2017). Both milk proteins and dietary polyphenols consist of a large group of molecules with complex structures, contributing both nutritive and additional health benefits, and forming complexes and/or conjugates via their natural affinity. Such molecules have a function in food structure formation and stabilisation. Thus, some structural, functional, and nutritional properties of the two components are, to some extent modified due to the existing format in products and the exposure manners to the digestive system being changed through their interactions. Desirable or undesirable, these changes have been found with the potential to be harnessed to fortify products with dietary polyphenols at a health-relevant dose with protein matrices, to create protein-polyphenol based novel ingredients.

1.2 Encapsulation methods

Spray-drying and freeze-drying were chosen as different encapsulation strategies for a comparison, to make protein encapsulates with unique physicochemical and nutritional properties. The novel protein encapsulates with different physicochemical properties possessed different application potentials on specific food matrix, providing more options for novel functional food development, and broadening the application of traditional protein

ingredients.

1.3 Spray-drier

The spray-drier 'Niro Mobile Minor™ 2000' (GEA, Germany) was used (located in the John Sutherland Teaching Lab, Canterbury University (Christchurch, New Zealand) (Figure 1.1a)). It has been designed and constructed as a research tool to handle small quantities of product. The process involves the atomisation of the feed solution into a spray of small sizes using a two-fluid atomiser powered by a compressed air supply. The droplets are exposed to a flow of hot air. As these droplets have a very large surface area, water evaporation takes place almost instantaneously within the short residence time of the drier. The droplets are transformed into dry powder particles, and then separated from the air using a cyclone. The dried powder particles are collected in a glass sample collection jar attached to the cyclone. The exhaust air is drawn out of the top of the cyclone, through the exhaust fan and ducting system, before being discharged to the atmosphere outside the building (Pisecký, 2020).

1.3.1 Freeze-drier

The research also used a freeze drier "Cuddon FD 5.5" (Cuddon Freeze Dry, New Zealand) (located at Riddles Lab, Lincoln University (Christchurch, New Zealand) (Figure 1.1b)). Freeze-drying is a low temperature processing technique with encapsulation effects, which has the potential to preserve sensitive components controlling deterioration of these compounds. In freeze-drying, the protein solution is frozen followed by removing water from the sample during sublimation (primary drying), and then the samples are dried again (secondary drying). The complexes and/or conjugates formed under an aqueous solution were solidified as what they are. It is an optimal drying process applicable to manufacture pharmaceuticals or biologicals that are unstable in an aqueous state for long periods of storage but that are stable in the dry state (Deepak & Iqbal, 2015).

1.3.2 Milk protein ingredients and their aqueous solutions

Whey protein is known as water-soluble in their native forms under a wide range of pH conditions (Sedaghat Doost *et al.*, 2020). Whey protein isolate has been widely accepted as a protein supplement and application on formulated foods due to its high protein content (> 90%), wide availability, and complete amino acids profile (Gorissen *et al.*, 2018; Turck *et al.*, 2019). In terms of other milk protein components, casein's water solubility depends on the pH conditions (Hinderink *et al.*, 2021). Sodium caseinate is one of the main commercial forms of casein-based protein ingredients. It has high protein content (> 90%), low fat content, low lactose content, with wide applications in food sectors, such as bakery, yogurt and ice cream, confectionery and chocolate, beverages. In this thesis, whey protein isolate aqueous solution (10%, w/w) was prepared to make the mixture of whey protein isolate and blackcurrant concentrate. Sodium caseinate aqueous solution (5%, w/w) was prepared to make the mixture of sodium caseinate and blackcurrant concentrate. The aqueous solution protein concentration we chose was partly out of concern for drying efficiency, encapsulation efficiency, and protein solubility.

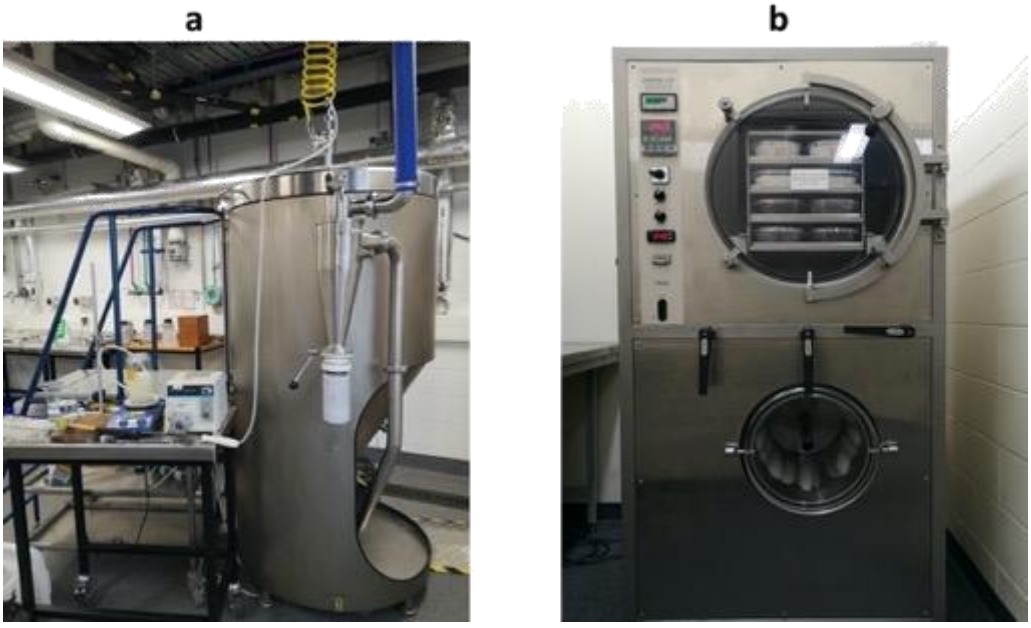


Figure 1.1 (a) Spray drier-Niro Mobile Minor™ 2000; (b) Freeze drier (Cuddon FD 5.5)

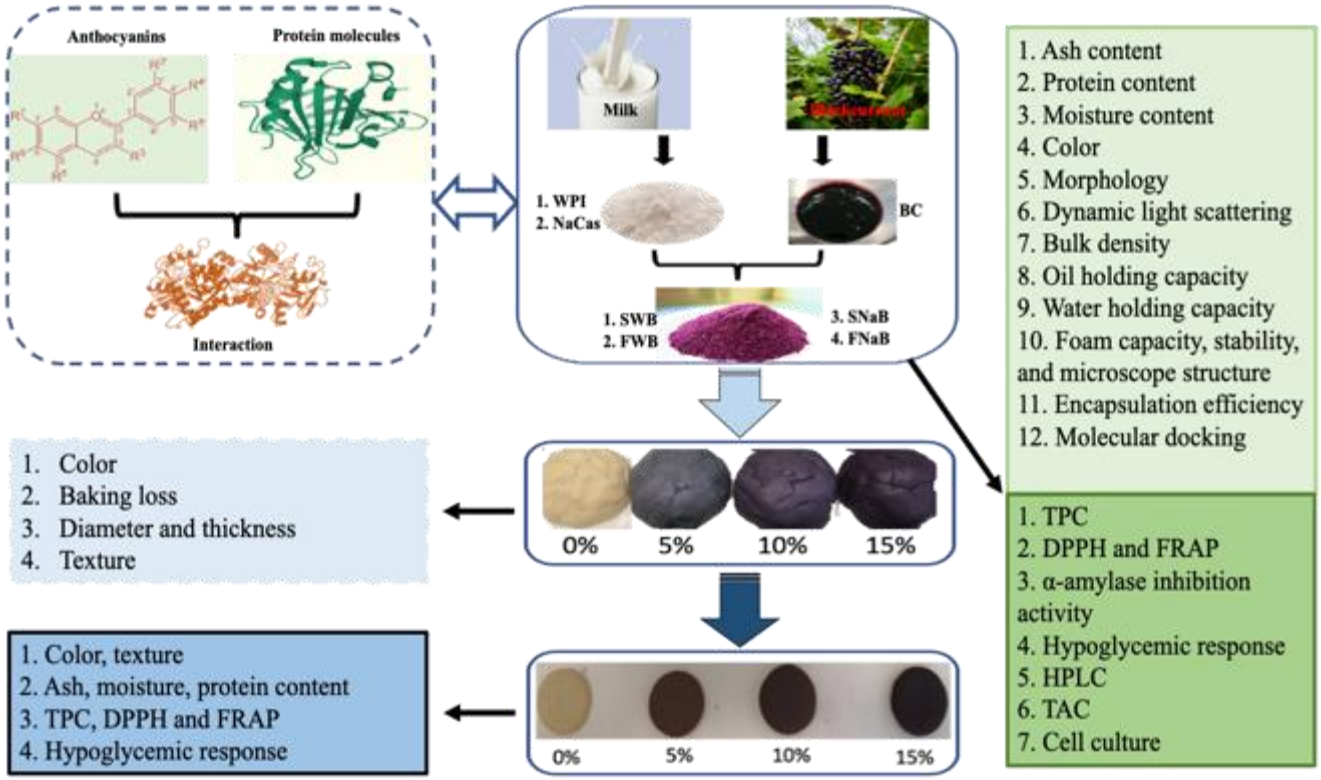
1.4 Objectives

- To develop novel protein ingredients by combining milk protein aqueous solution with blackcurrant concentrate, and forming encapsulates via spray-drying and freeze drying techniques
- To investigate and compare the physicochemical properties and nutritional characteristics of the novel protein ingredients produced by spray-drying and freeze-drying strategies
- To determine the functional properties and potential anti-cancer properties of the novel protein ingredients produced by spray-drying and freeze-drying strategies
- To incorporate the novel protein ingredients into practical food matrix (cookie), and further investigate the effects on food product physicochemical properties and nutritional value

1.5 Hypothesis

- Addition of blackcurrant concentrate will fortify the nutritional values of milk protein ingredients.
- Addition of blackcurrant concentrate will alter the functional properties of milk protein ingredients.
- Spray-drying and freeze-drying will generate particles with unique properties.
- Application of novel protein ingredients into cookie will improve the nutritional values of cookie, including higher protein content, stronger antioxidant capacity etc..

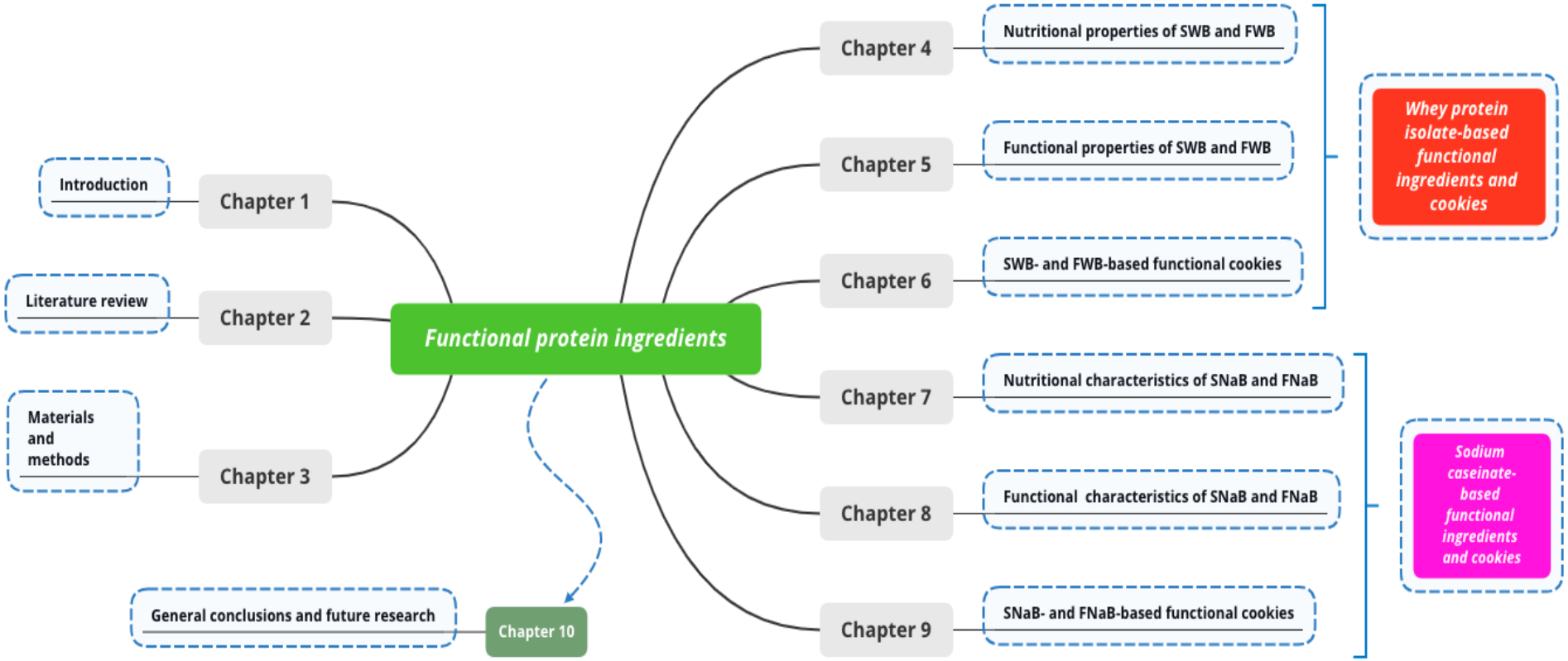
1.6 Research structure



Anthocyanin and protein molecule interact with each other at molecular level. WPI and NaCas were combined with BC at certain conditions, and obtained four different kinds of novel protein ingredients (SWB, FWB, SNaB, FNaB). The physicochemical, nutritional, and functional properties were well defined, followed by further application of these functional protein ingredients on cookies dough.

Abbreviation: WPI: whey protein isolate; NaCas: sodium caseinate; BC: blackcurrant concentrate; TPC: total phenolic content; TAC: total anthocyanin content; HPLC: high performance liquid chromatography; SWB: Spray-drying + Whey protein isolate + Blackcurrant concentrate; FWB: Freeze-drying + Whey protein isolate + Blackcurrant concentrate; SNaB: Spray-drying + Sodium caseinate + Blackcurrant concentrate; FNaB: Freeze-drying + Sodium caseinate + Blackcurrant concentrate

1.7 Thesis outline



SWB: Spray-drying + Whey protein isolate + Blackcurrant concentrate; FWB: Freeze-drying + Whey protein isolate + Blackcurrant concentrate.

SNaB: Spray-drying + Sodium caseinate + Blackcurrant concentrate; FNaB: Freeze-drying + Sodium caseinate + Blackcurrant concentrate.

Chapter 2

Literature review-Functionalisation of bovine protein by dietary phenolics

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2.1 Protein polyphenol interactions

2.1.1 Introduction

During the past decade, the functionalisation of milk protein with dietary phenolics has generated increasing attention of researchers possibly as health-conscious customers have been demanding healthier food products (Condict, Paramita, & Kasapis, 2019; Yildirim-Elikoglu & Erdem, 2018). Consumers' demands are shifting towards food products with high protein contents and added health benefits, but low fat and carbohydrates contents (Schneider, Esposito, Lila, & Foegeding, 2016). Food manufacturers produce food products with additional health benefits (Flores & Kong, 2017). Milk has long been safely utilised to nourish life for thousands of years (Haug, Høstmark, & Harstad, 2007). Following the development of new analytical techniques during the last century, milk components and their detailed physical and chemical properties have been revealed (Hoffman & Falvo, 2004). As a result, milk protein ingredients have become important, widely available, and inexpensive materials of our modern food industry due to their complete nutrition profiles and irreplaceable functionalities, such as foaming, thickening, emulsification, texture in food processing (Ali, 2019; Pessato *et al.*, 2018). Functionalisation of bovine whey proteins with dietary phenolics obtained from plant-based food sources has obtained increasing attention of researchers and consumers (Foegeding *et al.*, 2017). For instance, Tumbas Šaponjac *et al.* (2016) have developed a functional whey protein ingredient by the encapsulation of cherry pomace extract, and innovatively incorporated it into cookies to produce functional snacks.

Bovine casein-sourced ingredients, including α-casein, β-casein, κ-casein and caseinate, have been studied as carrier materials for sensitive dietary phenolics (Casanova *et al.*, 2018). Sodium caseinate micellar nanostructures or nanoparticles have displayed high entrapment efficiency and bio-protection for quercetin and curcumin (Ghayour *et al.*, 2019). Functional dairy foods, such as yoghurt enriched with fruit puree, and cheese pigmented by anthocyanins, have attracted customers' attention (Rashidinejad, Birch, & Everett, 2016).

Both bovine sourced whey proteins (Figure 2.1), bovine sourced casein ingredients (Figure 2.2) and dietary phenolics (Figure 2.3) are forms of isolated components or mixtures with complex chemical structures or components. Casein has been separated into individual components (α-casein, β-casein, κ-casein) or processed into protein ingredients (sodium caseinate, calcium caseinate). Whey has also been isolated into individual protein components (β-lactoglobulin, α-lactalbumin, bovine serum albumin, lactoferrin) or processed into protein ingredients (whey protein concentrate, whey protein isolate). Their interactions at molecular levels (bottom-up) are complicated due to structural differences, various binding mechanisms, environmental factors (pH and temperature), and disparate evaluation techniques (Considine, Flanagan, Loveday, & Ellis, 2020). By contrast, their combinations at mixture levels (top-down) are macroscopic with practical applications owing to the direct combination of mixture forms of food ingredients (Foegeding *et al.*, 2017).

A literature search indicated that the number of publications with the keywords "whey protein", "casein", and "dietary phenolic" has increased sharply in the last decade (2010-2020), revealing that more and more researchers have paid attention to this topic. This review summarises recent advances (2010 onwards) in the functionalisation of milk protein ingredients with dietary phenolics. Furthermore, this review outlines the influence of food processing, distribution or storage, and digestion processes on the physicochemical and functional properties of proteins as well as the bioavailability of dietary phenolics. Potential

future directions for research, and practical applications are also highlighted. The whole review structures are illustrated in Figure 2.4.

2.1.2 Whey protein and its fractions

Whey protein is mainly obtained from cheese processing by product-whey (Figure 2.2). It comprises of isolated individual components, including β-lactoglobulin, α-lactalbumin, bovine serum albumin, immunoglobulins and several other protein/peptide components, namely lactoperoxidase, lysozyme, and lactoferrin (Ben Ounis, Gauthier, Turgeon, Roufik, & Pouliot, 2008). Commercially produced whey protein usually comes in three major forms: whey protein concentrate (WPC) (29-89% protein by weight), whey protein isolate (WPI) (≥ 90%), whey protein hydrolysate (digestive enzyme-treated) (Athira *et al.*, 2015; EFSA, 2010). Owing to its high nutritional values, various functional properties, wide availability, and cost-effectiveness, whey protein is frequently utilised in formulated foods (Hoffman & Falvo, 2004).

2.1.3 Casein protein ingredients and individual components

Caseins, exist as micelles in raw milk, and consist of three fractions, namely α-casein, β-casein, κ-casein, all of which are of great interest in the food industry and food researchers (Livney, 2010; Ranadheera, Liyanaarachchi, Chandrapala, Dissanayake, & Vasiljevic, 2016). Excepting being processed into cheese products, casein is processed into caseinate of sodium or calcium, which has a wide application in food processing industry (Luo, Pan, & Zhong, 2015; Peng *et al.*, 2020). Casein has been processed into caseinates normally by resolubilising the acid casein precipitate by alkali to about pH 6.7 to cater further industrial application (Singh & Ye, 2014) or individual components (α-casein, β-casein, κ-casein). Each individual component or commercial protein ingredients has the potential to be used as a carrier for dietary phenolics with unique functional properties and nutritional profiles.

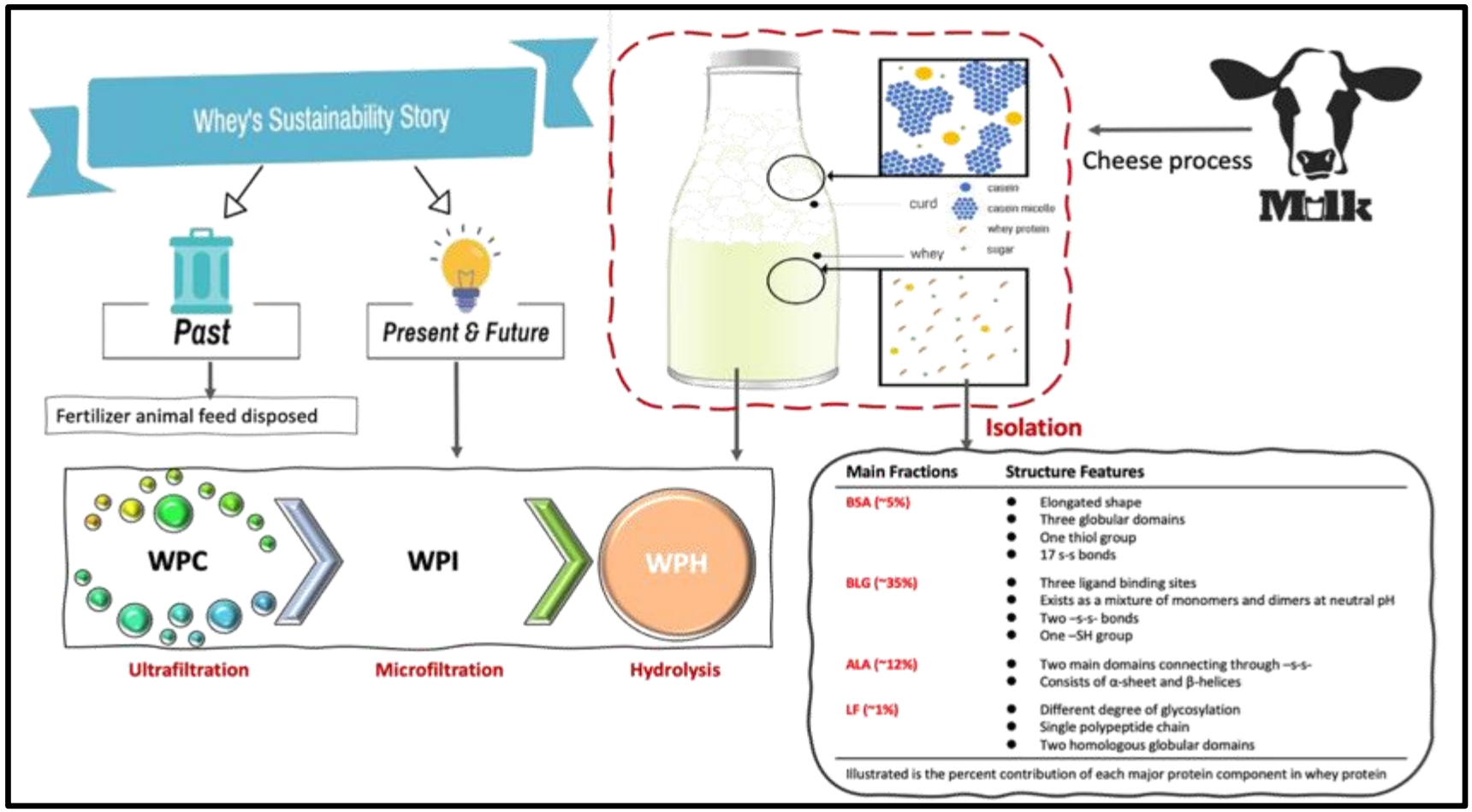

Figure 2.1 Scheme for the individual fractions and main commercial forms of whey

BSA: bovine serum albumin; BLG: ß-lactoglobulin; ALA: α-lactalbumin; LA: lactoferrin; WPC: whey protein concentrate; WPI: whey protein isolate; WPH: whey protein hydrolysate

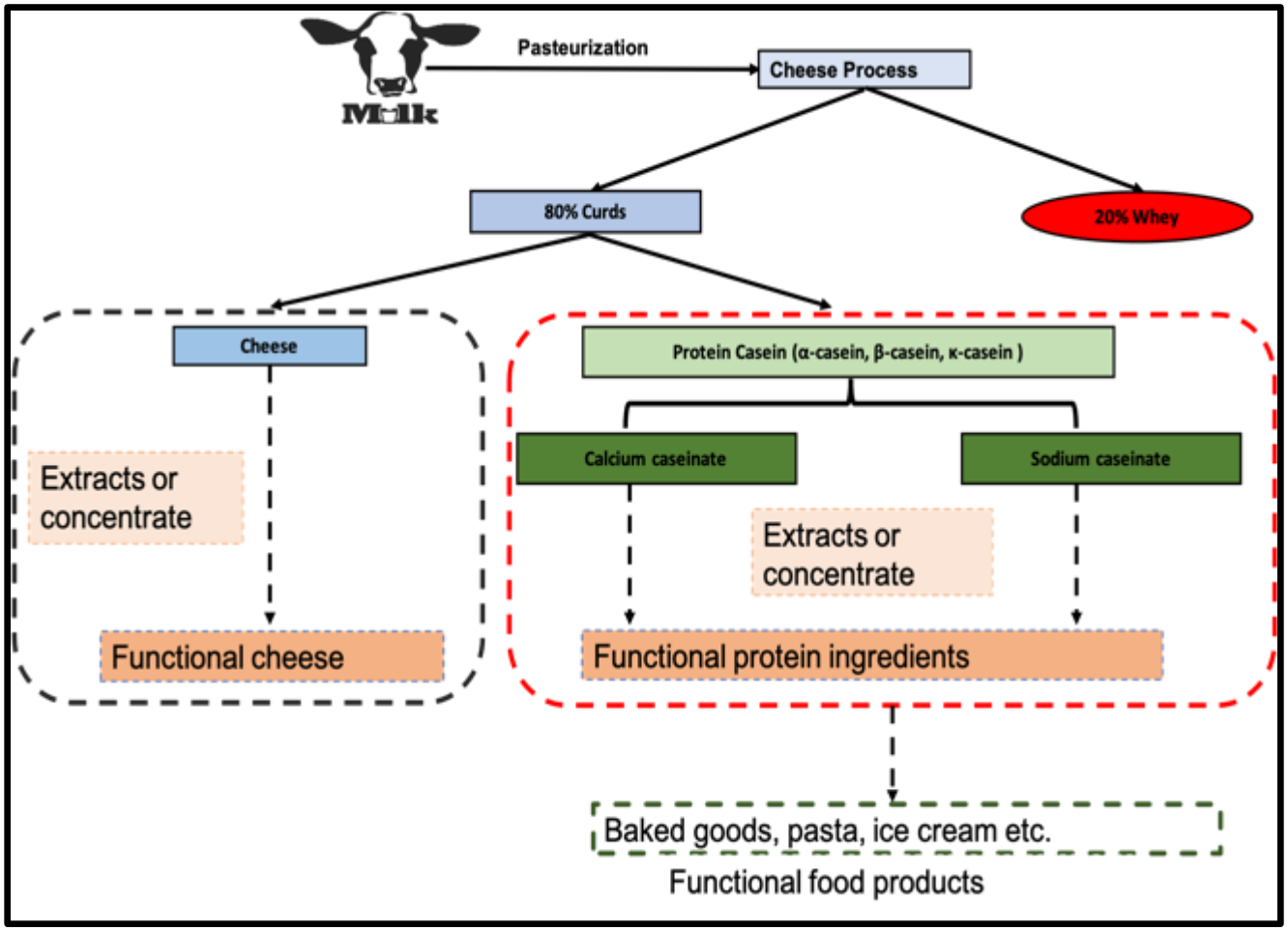

Figure 2.2 Scheme for the individual fractions and main commercial forms of casein and their combination with dietary phenolics

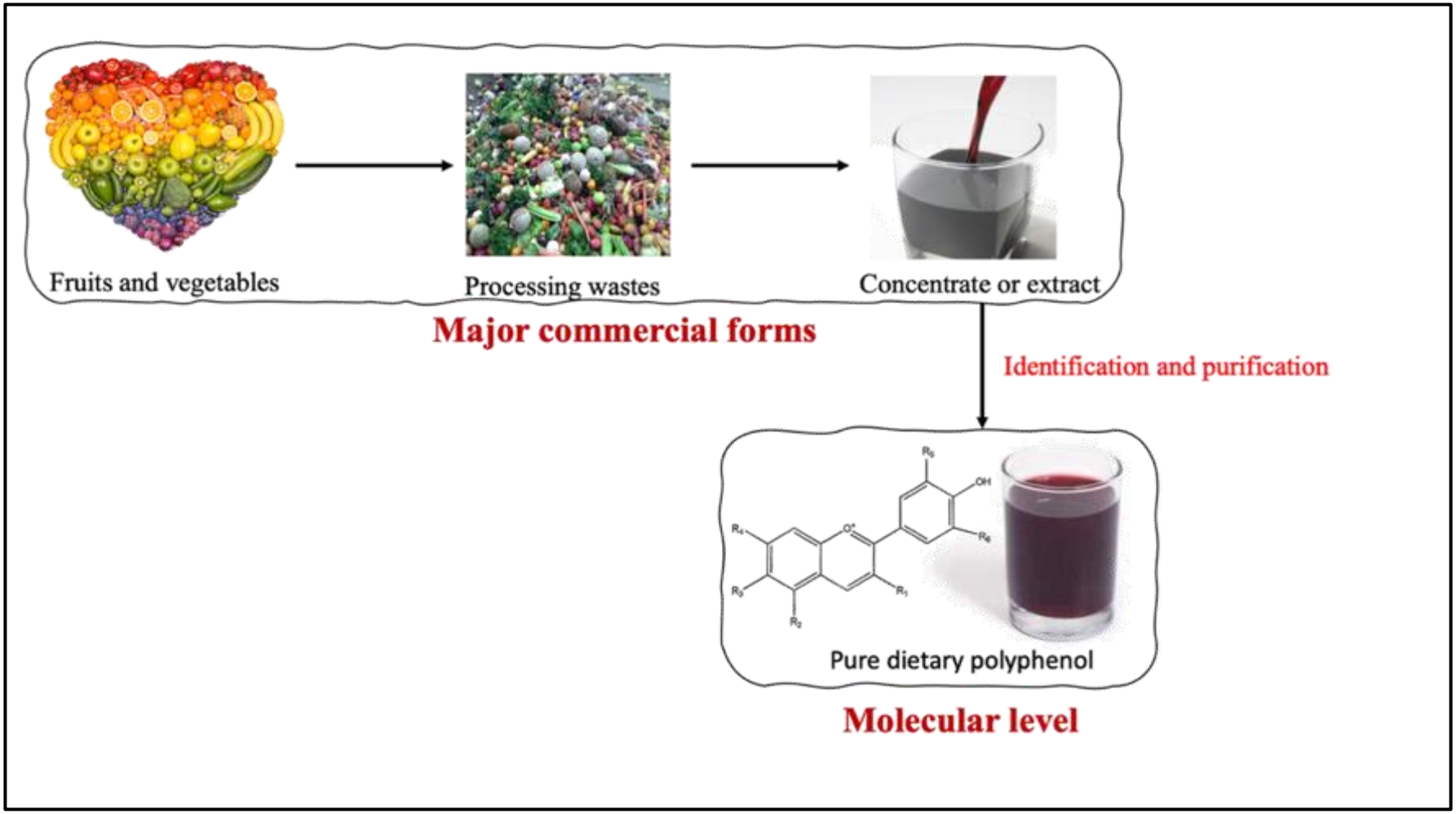

Figure 2.3 Dietary phenolics occurs in plant-based food resources, mainly existing in the forms of juice concentrate, extracts, and pure

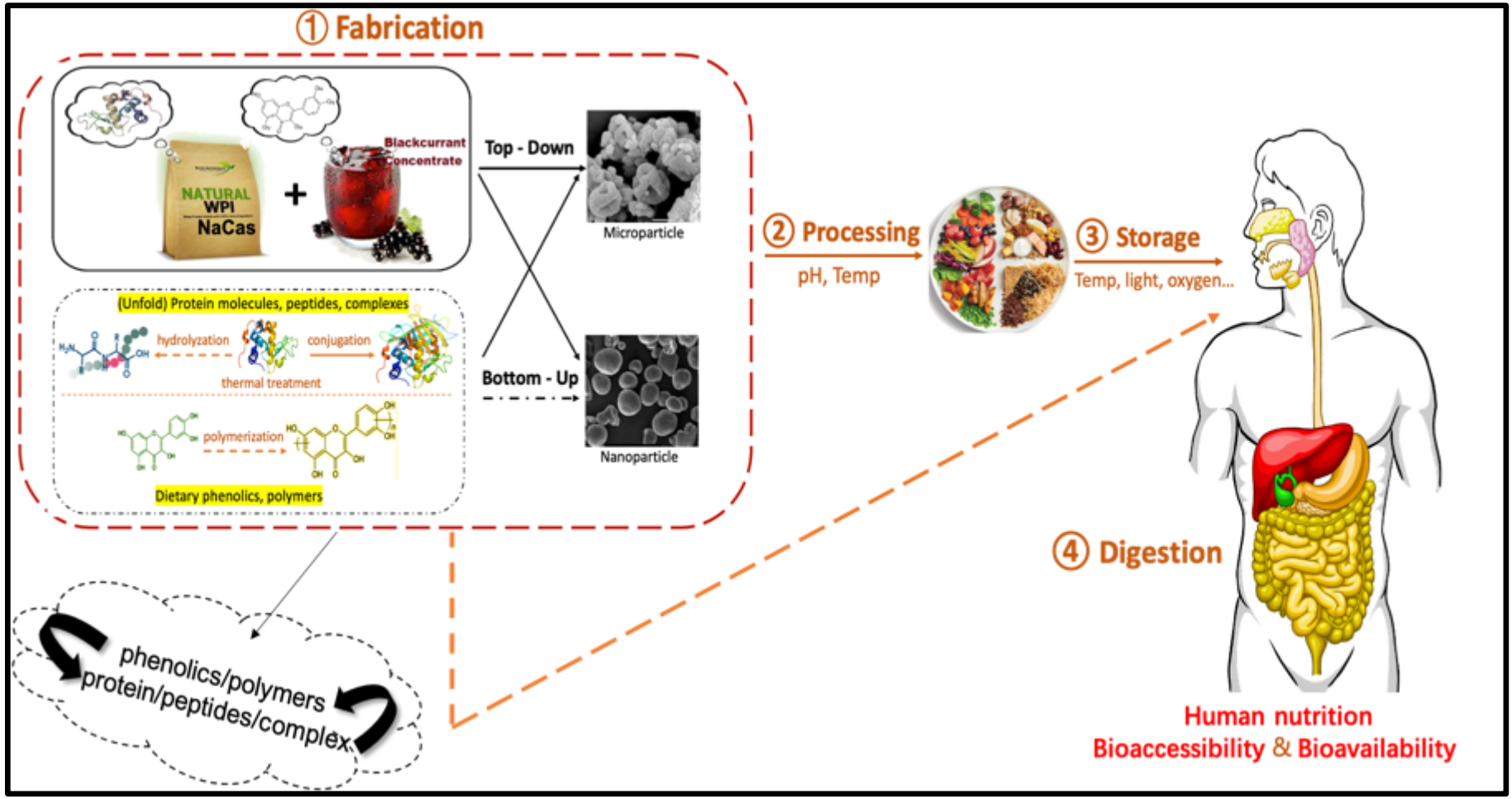

Figure 2.4 Graphic abstract for literature review section.

2.1.4 Common existing forms of dietary phenolics

Dietary phenolics are secondary metabolites generated by plants in response to their environmental stresses, and are especially abundant in some plant-based foods, such as fruits, tea, coffee, dark chocolate (Jakobek, 2015; Zagury, Kazir, & Livney, 2019; Zhang, Yu, Sun, Liu*, et al.*, 2014), with one feature in common: a hydroxyl group bonded to an aromatic ring (Ozdal, Capanoglu, & Altay, 2013). Dietary polyphenols have increasingly become an intense focus of the scientific community (Ozdal *et al.*, 2016), health conscious customers and food producers, due to both solid scientific evidence and epidemiological studies soundly approving that dietary polyphenols intake have various extra health benefits, such as anticarcinogenic properties (Ho, Thoo, Young, & Siow, 2019; Weng & Yen, 2012), cardiovascular disease protection (Mursu *et al.*, 2008; Tressera-Rimbau *et al.*, 2017), neurodegenerative diseases (Zhang & Tsao, 2016), type 2 diabetes (Xiao & Hogger, 2015), obesity (Meydani & Hasan, 2010). Such benefits are mainly via their antioxidant, anti-inflammation, and intestinal microbiota modulating properties (Cory *et al.*, 2018). Interestingly, they are not involved in growth and energy metabolism after consumption (Harnly, Bhagwat, & Lin, 2007), which, unlike other food components, cause no extra worries about calories after consumption. Moreover, some of them have been reported to be utilised as natural colourants, such as using anthocyanin in cheese production (Prudencio, Prudêncio, Gris, Tomazi, & Bordignon-Luiz, 2008). Commonly available forms of dietary phenolics (Figure 2.4) are concentrates (Bazaria & Kumar, 2017; Isik, Altay, & Capanoglu, 2018), extracts (Akdeniz, Sumnu, & Sahin, 2018; Xue, Su, Meng, & Guo, 2019; Yu & Ahmedna, 2013), and pure isolated components (Najgebauer-Lejko, Sady, Grega, & Walczycka, 2011).

2.1.5 Challenges in food application

There are a few challenges in terms of the practical applications of milk proteins. Milk proteins are still great allergen sources for allergic population (Restani *et al.*, 2004). Elimination of

allergenic food from the diet is the most common strategy but at the expense of losing important source of nutrients. Additionally, milk proteins are unstable during processing, storage and digestion process. To reduce the allergenic capacity and stabilise the proteins, the interaction with other molecules seems like a promising solution. It is a popular perception that whey protein is an allergen (Pessato *et al.*, 2018). The foaming ability of whey proteins is beneficial to foods with foam structures, but it can be detrimental to other applications such as beverage manufacturing (Guimaraes *et al.*, 2020).

Despite all these attractive advantages, dietary polyphenols also possess some drawbacks that severely limit their usage in food industry. First of all, there is a content of natural polyphenols in foods, which leads to underconsumption for populations. It has been reported that no more than 1% of the population of the United States population reaches the recommended level through daily consumption of fruits and vegetables (McGuire, 2013). Health benefits of polyphenols in the human body are dose dependent (Recommended Daily Intake ~1g/D) (Scalbert & Williamson, 2000), which means that only after being absorbed into the circulation system at an effective concentration, do appropriate bioavailable forms exist (Lila *et al.*, 2017), and these polyphenols can then exert their functions. The low solubility and instability, especially of phenolic components in free form after extraction (Jia, Dumont, & Orsat, 2016), under conditions encountered in food processing (temperature, ionic strength) and storage (pH, temperature, oxygen, light), or in the gastrointestinal tract (pH, enzymes, presence of other nutrients) limit their applications, biological activity and potential health benefits, sometimes giving unpleasant flavour, unwanted colour change, and diminished sensory acceptation (Rossetti, Bongaerts, Wantling, Stokes, & Williamson, 2009). Thus, protecting polyphenols with carriers instead of administering those as free form can increase the concentration and control the release, concurrently, overcoming the inherent limitations (Ho *et al.*, 2019).

2.2 Combination of milk proteins with dietary phenolic

Fortunately, the challenges mentioned in 2.1 can be alleviated via the combination of dietary phenolics with whey protein under specific conditions and routine methods (Kori, Mahesar, Sherazi, Laghari, & Panhwar, 2020). Practically, the interaction of milk protein with dietary phenolics is inevitable in some of the milk-based products (Guimaraes *et al.*, 2020), which is directly related to the product stability, texture and nutrition (Eyiz, Tontul, & Turker, 2020). Therefore, understanding the interactions of milk protein and dietary phenolics may foster the resolution of these practical issues. There is considerable interest in utilising whey proteins and sodium caseinate as carriers for reactive dietary phenolic components in the food sector (Hoffman & Falvo, 2004). Substantial evidence has suggested that milk protein may act as a carrier of plant polyphenols and improve the intestinal absorption of phenolic compounds (Meydani & Hasan, 2010). Whey protein is an efficient wall material due to its wrapping and encapsulation effects (Khalifa, Nie, Ge, Li, & Li, 2018). Casein is especially effective in encapsulating some hydrophobic molecule due to the formation of micelles structure in aqueous (Yan, Hu, & Yao, 2009). Thus, based on systematic understanding of interactions of milk protein with dietary phenolics, fabricating and functionalising whey protein and caseinate are promising strategies to develop novel protein materials (particles, films, emulsion systems, and hydrogels), which potentially possess all inherent advantages of each component as well as alleviate or avoid their challenges in practical applications mentioned above (Horincar *et al.*, 2019).

2.2.1 Binding mechanisms at molecular levels-quantitative research

Non-covalent complexation and covalent conjugation are two categories of fundamental mechanisms for individual protein components and isolated pure polyphenols interactions, which are determined by unique amino acids sequences, the number of amino acid residues

(especially proline residues), -SH groups, -S-S- groups, the different native spatial conformations, molecular weights, and structures of components (Ranadheera *et al.*, 2016). Non-covalent physical interactions, including electrostatic, hydrophobic, van der Waals and hydrogen bonding, occur spontaneously and form reversible weak complexes. Whereas covalent bindings are less frequent, these result in the formation of irreversible stable conjugates (Le Bourvellec & Renard, 2012; Liu *et al.*, 2019), which is more favourable in terms of protein functionalisation. Liu *et al.* (2019) reported that covalent zein-polyphenol complexes had higher thermal stability than their non-covalent counterparts. Liu *et al.* (2019) summarised the artificial techniques, including alkaline treatment, free radical-mediated grafting, enzyme-catalysed grafting, and chemical coupling, to form stable phenol-protein conjugates. Harsh thermal conditions and pH variations are two critical factors that affect the interactions of protein and polyphenols during food processing, storage, and human digestion (Prudencio *et al.*, 2008; Yildirim-Elikoglu & Erdem, 2018). Other factors, such as ionic strength, co-existing components, and the ratios of protein-polyphenol have also been considered in recent studies (Stanciuc *et al.*, 2017).

2.2.2 Encapsulation strategies at mixture levels-synergistic effects

Encapsulation methods, including spray-drying or freeze-drying, hydrogel formation, emulsification, and film formation, are the most common strategies for the fabrication of protein-based dietary phenolics delivery systems (Liu, Ma, Gao, & McClements, 2017). The formation of complexes and/or conjugates using whey protein can protect dietary phenolics from oxidation and degradation during shelf-life, or while passing through the gastrointestinal (GI) tract, and these complexes assist more intact polyphenols to arrive at their absorption sites (Jakobek, 2015). Changes in the conformation and secondary structure of caseins was observed between the interactions of polyphenols with casein. Anthocyanins and β-casein interaction was found resulting in changed protein structure (Wei *et al.*, 2018). The binding of

cyanidin-3-glucoside slightly modified the average diameter of sodium caseinate nanoparticles without alteration of its surface charge suggesting a complexation of cyanindin-3-glucoside molecules in the internal casein structure. Thus, sodium caseinate constitutes a putative nanocarrier for anthocyanins in new functional foods (Casanova *et al.*, 2018).

In order to further improve their stability, the liquid solution can be turned into powders for better ease of handling as a bioactive preparation (Kreatsouli *et al.*, 2019; Xue *et al.*, 2019). Other strategies, including ionisation, polymerisation, enzyme treatment, thermal treatment, alkaline conditions, and chemical linkers, and these have been reported to assist with fabrication (Dhayal, Sforza, Wierenga, & Gruppen, 2015; Fan, Liu, Gao, Zhang, & Yi, 2018; Quan, Zhang, Zheng, Lu, & Lu, 2018; Tantoush *et al.*, 2011). The binary (protein and phenolics) or ternary systems (protein, polysaccharide, and phenolics) might be attractive forms to use in order to develop novel food-grade materials with better physicochemical properties by delivering high protein and extra health benefits simultaneously (Rashidinejad *et al.*, 2016), with the potential to be used as a substitute for fat or carbohydrates based on the soft matter theory (Schneider *et al.*, 2016).

2.2.3 The gap between molecular levels interactions and mixture levels combinations

The insights from molecular level interactions cannot be applied to the mixture level combinations due to the interference of other co-existing components. Understanding the complexities or conjugations at the molecular level provides valuable knowledge for the quantitative production and distribution of beneficial phenolics for health. Similarly, understanding the combinations at the mixture level provides valuable knowledge for the synergistic use of beneficial phenolics for wellbeing.

2.2.4 Research Methodology differences

Research methods, covering intermolecular interactions, microstructures, macrostructures, and physicochemical, functional, and nutritional properties, have recently been summarised by Czubinski and Dwiecki (2017). Multiple spectroscopic techniques were widely applied for different purposes based on the intrinsic fluorescence properties and native structures of proteins. Fourier Transform Infrared Spectroscopy and Circular Dichroism have been used as complementary techniques to study the changes in protein secondary structures. Jia, Gao, Hao, and Tang (2017) highlighted that the quenching ability on the intrinsic fluorescence intensity depends on the number and location of hydroxyl groups in polyphenols. A molecular modelling approach has been used to bring atomic-level details on the interactions of whey protein with phenolics. Molecular dynamics simulations and molecular docking studies have been used to provide details on the affinity and binding sites of β-lactoglobulin to cyanidin 3-rutinoside, one of the major components of anthocyanin extract from sour cherry (Tantoush *et al.*, 2011). In contrast, in terms of mixture level of combinations, studies have been focusing on their macroscopic properties, such as colour profile (Khalifa *et al.*, 2018), morphology (El-Messery, El-Said, Demircan, & Ozçelik, 2019), and bulk density (Tsali & Goula, 2018).

2.3 Delivery systems

Based on the available forms, including isolated individual components (β-lactoglobulin, α-lactalbumin, bovine serum albumin, lactoferrin) the structure of the leading commercial forms of whey protein ingredients (whey protein concentrate, whey protein isolate and whey protein hydrolysate), are illustrated in Figure 2.2, and dietary phenolics (purified compounds, concentrates, and extracts) are illustrated in Figure 2.4. The functionalisation of whey proteins by dietary phenolics can be classified as follows:

I. A single-molecule level complexation or conjugation, giving a deeper understanding of their interactions at molecular levels.

II. Whey protein isolate as a carrier for a model polyphenol, functionalising whey protein isolate by a specific model polyphenol, (whey protein isolate was set as a special category due to its high protein content).

III. Bulk materials (mixtures) complexation or conjugation, forming dietary phenolic-fortified whey protein ingredient.

Similarly, the interactions among casein proteins (α-casein, β-casein, κ-casein) (Figure 2.3) and dietary phenolics (Figure 2.4) can be described at a molecular level and mixture level, respectively. Specifically, individual casein components can be utilised as carriers for pure polyphenol components to uncover their interaction behaviours at a molecular level. Caseinate can be used as wall materials to encapsulate extract or concentrate full of bioactive compounds.

2.3.1 A single-molecule level complexation and/or conjugation

2.3.1.1 Model proteins (β-lactoglobulin, α-lactalbumin, bovine serum albumin, lactoferrin) from whey and model protein (α-casein, β-casein, κ-casein) from casein

Individual whey components, including β-lactoglobulin, α-lactalbumin, bovine serum albumin, lactoferrin, have normally been chosen as model proteins to uncover the interaction mechanisms of whey protein and dietary phenolics (Figure 2.2). Bovine serum albumin has been widely reported as a "target" of therapeutically active phenolics, and closely related to phenolic metabolism. Thus, a considerable number of researchers have focused on the interactions between bovine serum albumin and phenolic acids (Arancibia-Avila *et al.*, 2011; Engstrom *et al.*, 2016; Gorji *et al.*, 2015; Kim *et al.*, 2019; Poor *et al.*, 2018; Zhang, Yu, Sun, Guo*, et al.*, 2014). It is the structural homology with human serum albumin, which is the most abundant drug transporter in serum (Shi, Zhang, Chen, & Peng, 2011). The interactions between bovine serum albumin and a small molecule result in a stable protein drug complex, which may be considered as a model for gaining bioactive compounds. Therefore, much

information concerning storage, disposition, mechanism, pharmacokinetics and toxicity could be obtained by analysing the complexes of model phenolics with bovine serum albumin (Liu, Ma, Chen, Xiong, & Shi, 2012).

Accounting for 50-55% of total whey protein, β-lactoglobulin has been the most widely studied as a natural transport agent for purified dietary polyphenols (El-Maksoud *et al.*, 2019; El-Maksoud *et al.*, 2018; Li, Cui, Ngadi, & Ma, 2015; Liang *et al.*, 2016). It belongs to the lipocalins family, which means β-lactoglobulin has an exceptional capability to deliver small hydrophobic molecules, such as dietary phenolics, and interact with cell surface receptors (Ș. A. Milea *et al.*, 2020). However, it has been recognised as one of the major milk allergens (Taheri-Kafrani *et al.*, 2009). Enzymatic processing by laccase and the employment of sour cherry phenolic extract as the mediator of enzymatic reaction may improve β-lactoglobulin safety and availability of peptides following the digestion by pepsin, while conserving its bioactivity (Tantoush *et al.*, 2011).

Alpha-lactalbumin is a vital dairy protein ingredient with its well-known nutritional values and potential biological functions (Wang *et al.*, 2014). It has strong calcium-binding properties and is widely found in the milk of humans and the milk of other mammals (Jiang *et al.*, 2020). Alpha-lactalbumin can bind with hydrophobic polyphenols such as oleuropein (Katouzian, Jafari, Maghsoudlou, Karami, & Eikani, 2020) and resveratrol (Cheng, Fang, Bakry, Chen, & Liang, 2018). It is also proposed as an ideal protein for usage in medical applications due to its anti-microbial and anti-tumour activities. Proteolytic digestion of α-lactalbumin leads to the formation of three peptide chains bearing antibacterial properties that hinders the proliferation of GI tract bacteria (Katouzian *et al.*, 2020).

Compared to other whey protein components, lactoferrin, a protein component from liquid whey, has been studied to a lesser extent on the interaction with phenolics due to its less quantitative importance (Rezende *et al.*, 2019).

The interaction of anthocyanins and β-casein result in a change in protein structure (Wei *et al.*, 2018). Static quenching was observed in different combinations, such as anthocyanins and β-casein. The number of binding sites was recorded as an important parameter reflecting the interactions. The interaction between anthocyanins and β-casein showing one binding site, indicating that a molar ratio of nearly 1: 1 combination would give the best binding efficacy (Wei *et al.*, 2018). Hasni *et al.* (2011) found that tea polyphenols can bind to α- and β-casein with both hydrophilic and hydrophobic interaction but hydrophobic binding prevails. Several amino acid residues were involved in the association.

2.3.1.2 Common purified and studied polyphenols

Among polyphenols, the most commonly studied are catechins, tannic acids, caffeic acid, oleuropein, and rosmarinic acid, all of which can be found in our daily foods in substantial content (tea, coffee, vegetable, and fruits) (El-Maksoud *et al.*, 2018; Flores & Kong, 2017; Ishtikhar *et al.*, 2018; Kim *et al.*, 2019).

2.3.1.3 pH and temperature

Two critical factors affecting protein and phenolic interactions (pH and temperature) have been defined in recent publications. Liubchak, Lawrence, Holness, and Price (2020) suggested that the pH of such combinations should be in between the range of 2.0-7.5 to meet physiological pH ranges. The authors also reported that pH conditions could modulate the binding forces between β-lactoglobulin and tea catechins via hydrophilic (Van der Waals Forces or hydrogen bonding) and hydrophobic interactions, thus affecting the efficacy of phenolics. The published temperature ranges were below 50℃, with the primary concern of the thermal sensitivity of both protein and dietary phenolics (Xu *et al.*, 2019). However, food production, storage, and digestion procedures, lead to different pH conditions, such as gastric and intestinal pH, and harsh temperature conditions during sterilisation and food processing. Zhang, Wang, Xu, and Hu (2017) concluded that binding force was more potent at lower pH

value when β-lactoglobulin interacted with methyl gallate, epigallocatechin gallate, and epigallocatechin. Recently, literature reported that thermal treatment with β-casein and ferulic acid at ultra-high temperature level induced the formation of covalent bond, which was opposed to the traditional non-covalent interactions being reported consistently for food matrices at ambient temperatures and in non-alkaline conditions (Condict, Kaur, Hung, Ashton, & Kasapis, 2019). Thus, the processing temperature can be manipulated to avoid or reinforce covalent interaction between protein and polyphenols for optimisation of functionality in new product concepts.

2.3.1.4 Binding affinity

Various environmental factors, including chemical bonds, metal ions, and functional groups, have been studied for affecting protein-polyphenol combination strength. The non-covalent interactions are reversible, which might be disturbed by various environmental conditions. By contrast, covalent complexation is irreversible, which could be a possible manner to generate relatively stable complexes (Czubinski & Dwiecki, 2017). Calcium is a common ion existing in dairy products, which has a benefit for the formation of β-lactoglobulin with β-lactoglobulin network upon heating in β-lactoglobulin gelation. Jia *et al.* (2017) reported that the existence of calcium in the system might harm β-lactoglobulin phenolic binding ability by competing with the limited binding sites. Functional groups in phenolics have a direct relationship with the affinity between β-lactoglobulin and bovine serum albumin. Ozdal *et al.* (2013) concluded that the number, and the position, of substituted hydroxy, methoxy, and methyl groups on the ring of phenolic acids and their derivatives could influence the binding affinities of β-lactoglobulin. One of the potential mechanisms for increased binding affinities could be the involvement of the hydrogen bond in the interactions between the phenolic acids and β-lactoglobulin. Zhang *et al.* (2017) indicated that the high binding affinity of epigallocatechin gallate to β-lactoglobulin was due to the galloyl functional groups. Epigallocatechin gallate was

more effective than caffeic acid in the formation of β-lactoglobulin-polyphenol conjugates (El-Maksoud *et al.*, 2018). El-Maksoud *et al.* (2019) reported similar conclusions that in addition to molecular weight, structural features such as the number of galloyl groups and degree of oxidative coupling between the galloyls, positional isomerism and cyclic vs. acyclic glucose core were the major structural features affected the ability of the monomeric hydrolysable tannins to form insoluble complexes with bovine serum albumin. It has been found that non-covalent interactions could lead to partial conformational alterations in the secondary structure of proteins, increased in α-helical content with decreased in β-sheet, random coil, and other structures (Ishtikhar *et al.*, 2018), thus resulting in the complexes with increased thermal stability.

2.3.1.5 Artificial creation of covalent bonds

Both covalent, and non-covalent, bonds can synchronise spontaneously. Phenolics can be oxidised to their corresponding quinones and semiquinones, which can further undergo covalent reactions with an enormous number of nucleophiles such as cysteine or lysine on the protein (Rohn, 2014). Covalent interactions are much more valuable when considering the application of the complexes due to the stability differences. Recently chemical linkers, such as carbodiimide (El-Maksoud *et al.*, 2018), tetra ethylene glycol (El-Maksoud *et al.*, 2019), and EDC/sNHS (Li, Pan, Yang, Rao, & Chen, 2019) were reported to catalysis to conjugate a natural antioxidant into β-lactoglobulin via covalent interactions with superior stability. El-Maksoud *et al.* (2019) found that crafting caffeic acid onto a polymer by stretching could be a better strategy for inhibiting lipid oxidation than direct covalent bonding of amino residues in β-lactoglobulin-caffeic acid. While oligomers were superior to monomers in their capability to precipitate the model protein, their activities mostly depended on their size and overall flexibility. Artificially created covalent bonds between proteins and polyphenols opened a new route to design functional materials with enhanced functionality and biocompatibility, which

provided us with broad applications in pharmaceutics, cosmetics, and food processing (Wilson, Gasparini, & Matile, 2014).

2.3.1.6 Enhancement of antioxidant capacity

Comparing the antioxidant activity of whey protein with whey protein polyphenol complexes, complexation could increase antioxidant activities of whey protein to a certain extent (Ishtikhar *et al.*, 2018). However, the antioxidants could be further strengthened by the polymerisation of phenolic components. El-Maksoud *et al.* (2019) grafted polymerised caffeic acid onto protein. The extension of the polymer arm gave the coupling superior antioxidant properties, thus fully exposing the caffeine molecules to the water phase and scavenging free radicals.

2.3.1.7 Practical applications of encapsulates

The development of complexes has the potential to be utilised as a drug delivery tool with health benefits. Researchers have found that the binding affinity of bovine serum albumin with tannic acid or β-lactoglobulin with tannic acid was more significant at acidic pH, which resulted in the potential utilisation of these complexes for gut-targeted drug delivery (Xie, Wehling, Ciftci, & Zhang, 2017). Additionally, Wu *et al.* (2018) reported that when β-lactoglobulin conjugated with epigallocatechin gallate and caffeic acid by reducing IgE-binding capacity, the allergenicity of β-lactoglobulin would decrease, resulting in the production of hypoallergenic foods. However, the utilisation of purified phenolics, toxicology data and effective doses still needs to be taken into consideration (Granato, Mocan, & Camara, 2020).

2.3.2 Whey protein isolate combined with model polyphenols

Recent studies have shown great interest in utilising whey protein isolate as a protein source to investigate the interactions with some common purified polyphenols due mainly to whey protein isolate's high protein content (≥ 90%) and excellent solubility in a wide range of pH values, versatile functional properties as well as a broad range of food applications (Caporaso,

Genovese, Burke, Barry-Ryan, & Sacchi, 2016). Increased antioxidant strength and increased protein digestibility were consistent observations in recent studies on whey protein isolate and polyphenol complexes (Fan *et al.*, 2018; Guimaraes *et al.*, 2020; Zagury *et al.*, 2019).

2.3.2.1 Strategies to enhance interactions between whey protein isolate and polyphenols

Strategies including thermal treatments (Cao & Xiong, 2017), alkaline conditions, and enzymatic treatments (Ali, Keppler, Coenye, & Schwarz, 2018) have been widely investigated for the interaction of modified whey protein isolate with purified dietary polyphenols by comparing with natural whey protein isolate. The interactions of both modified and natural whey protein isolate with purified dietary polyphenols can alter the chemical, structural, and functional properties of the proteins as well as their effects on the biological functions of polyphenols.

Both alkaline conditions (pH = 9), and enzyme treatments, have proved to be useful strategies to form covalent bonds between whey protein isolate and specific polyphenols, which have resulted in functional protein ingredients with higher thermal stability (Ali, 2019; Ali *et al.*, 2018; El-Maksoud *et al.*, 2018). In addition, the interactions have also obviously altered some other physicochemical properties of proteins. Ali *et al.* (2018) have reported that the amount of free amino and thiol groups, as well as tryptophan content in whey protein isolate, decreased at alkaline (pH = 9) or enzyme treatment condition after covalently complexing with Rosmarinus acid. Similar conclusions were given by Ali (2019) regarding the interactions of whey protein isolate with different polyphenol compounds under alkaline conditions (pH = 9). Thermal treatments could modify the whey protein isolate structure by exposing more available binding sites and hydrophobic regions. Cao and Xiong (2017) studied the interaction mechanisms of natural whey protein isolate and heat-treated whey protein isolate with different types of pure phenolics. Oancea *et al.* (2017) reported that pre-treatment by heat treatment improved the encapsulation efficiency of β-lactoglobulin with anthocyanins since

the surface hydrophobicity increased sharply. However, modifications to other functional properties, such as foaming, emulsifying, water holding, and oil absorption, have not been investigated so far.

2.3.2.2 Allergenicity reduction

Bovine milk allergy is a severe public health issue with practical implications for the food industry. Whey protein, β-lactoglobulin, α-lactalbumin, and bovine serum albumin are involved in milk allergy (Restani *et al.*, 2004). The complexation of whey protein with caffeic acid and epigallocatechin gallate could be a promising strategy to reduce the allergenicity of whey protein (Pessato *et al.*, 2019; Pessato *et al.*, 2018). For example, galloyl groups in epigallocatechin gallate could act as a linker between whey protein molecules, resulting in modified molecular structure, and reducing allergenicity of β-lactoglobulin and bovine serum albumin by masking IgE-binding sites. The combination of polyphenols with allergenic milk protein provides a new way to address the problem of milk allergy.

2.3.3 Potential application of encapsulation technologies

This section summarises the combination of commercial whey protein ingredients (whey protein concentrate or whey protein isolate or whey protein hydrolysate) with dietary phenolics (concentrates or extracts) via various encapsulation strategies, to deliver synergistic effects of dietary phenolics to the food system and widen the application of whey protein ingredients and dietary phenolics.

2.3.3.1 Comparison of the study frequency of utilising different whey protein ingredients as carriers for dietary phenolics

Researchers have studied the functionalisation of whey protein isolate by various types of extracts or concentrates (Eyiz *et al.*, 2020; Sadeghi, Madadlou, & Yarmand, 2014; Zhang, Fan, Li, Chen, & Liang, 2019). Whey protein concentrate has been studied to a less extent, mainly due to the consideration of low protein content (Yadav, Bajaj, Mandal, & Mann, 2020).

Interestingly, rare studies reported the use of whey protein hydrolysate as wall materials to reflect the interactions of whey protein peptides with dietary phenolics and their synergistic biological activities (Madadlou & Abbaspourrad, 2018). Since whey protein can be hydrolysed by different enzymes, such as proteinase K (Madadlou & Abbaspourrad, 2018), thermolysin (Condurache *et al.*, 2019), trypsin (Mohamed *et al.*, 2019), and pronase E (Raikos, Hays, Stead, & Ni, 2019), which makes it possible to produce different peptides, which could possess some novel biological activities. For example, Mohamed *et al.* (2019) found that hyperglycaemia and dyslipidaemia are affected by mixing whey protein hydrolysate with fenugreek sprouts juice, barley sprouts juice, and cell-free probiotic extract.

2.3.3.2 Caseinate with dietary phenolics

The binding of cyanidin 3-O-glucoside slightly modified the average diameter of sodium caseinate nanoparticles without alteration of its surface charge suggesting a complexation of cyanidin 3-O-glucoside molecules in the internal casein structure. Thus, sodium caseinate constitutes a putative nanocarrier for anthocyanins in new functional foods. Sodium caseinate has been utilised to form nanocomplexes with tannic acid, revealing that interactions between proteins and polyphenols diminished the foam formation, but improved foam stability (Zhan *et al.*, 2018). Sodium caseinate-based foams and emulsions system with the addition of grape seed and green tea sourced flavonoid rich extracts have the potential to change the structure and stability of the protein-based system and on bioaccessibility of fortified flavonoid extracts (Elegbede, Li, Jones, Campanella, & Ferruzzi, 2018). Casein-chlorogenic acid binding and structural changes led to a significantly improved solubility, foaming capability, and foam stability, caffeic acid promoted the digestion of casein into small peptides (Jiang, Zhang, Zhao, & Liu, 2018).

2.3.3.3 Encapsulation and co-encapsulation

Encapsulation is defined as the process of coating materials attached to the surface of core materials to form small capsules to prevent unstable compounds like phenolics from various environmental conditions. Macromolecular substances, such as protein, carbohydrate, and fat, either used as single ingredients or in combinations, perform as wall materials as they possess thermal and mechanical stability to protect the sensitive compounds through variable conditions (Akdeniz *et al.*, 2018). Encapsulation makes molecular structures more stable thanks to the wall material acting as a physical permeability barrier for preventing the diffusion of molecular oxygen. As a result, the shelf-life of the encapsulated products may be prolonged (Tsali & Goula, 2018). Co-encapsulation, focusing on its synergistic effects, is defined as the simultaneous encapsulation of several compounds with a single coating material. For instance, blueberry concentrate has been shown to be more effective in bioaccessibility and nutritional benefits than single purified components (Bamba *et al.*, 2018). Similarly, Colin-Cruza, Pimentel-Gonzalez, Carrillo-Navas, Alvarez-Ramirez, and Guadarrama-Lezama (2019) co-encapsulated blackberry juice and *Lactobacillus acidophilus* with whey protein concentrate by spray-drying strategy to improve its functionality.

2.3.3.4 Encapsulation strategies

Spray-drying (Tsali & Goula, 2018), freeze-drying (Akbas *et al.*, 2017), emulsion (Busic *et al.*, 2018), film (Talon, Vargas, Chiralt, & Gonzalez-Martinez, 2019), and hydrogel techniques (Betz *et al.*, 2012), have been applied for the encapsulation of whey protein-based phenolics. Based on our literature research, more than half of the reported whey protein isolate encapsulation processes have been conducted by using spray-drying strategy. Kreatsouli *et al.* (2019) highlighted that the spray-drying inlet/outlet temperature has an effect on antioxidant activity. El-Messery *et al.* (2019) indicated that whey protein secondary structure changes during spray-drying might be related to improved encapsulation stability. Therefore, the

control of spray-drying parameters is of vital importance for the attributes of obtained particles. However, there is a lack of research dealing with the evaluation of combined encapsulation techniques for the delivery of naturally derived polyphenols. Busic *et al.* (2018) illustrated that a combination of emulsion processing with spray-drying could be a promising strategy for making food ingredients stable. However, this process was not reported extensively so far.

2.3.3.5 Binary wall materials and the ratio of the mixtures

Binary mixtures of proteins and carbohydrate wall materials, especially whey protein with maltodextrin, improve the stability and antioxidant capacities of polyphenols. Recently, polysaccharide-protein complexes have been reported as promising resources for the encapsulation of polyphenols (Milincic *et al.*, 2019). Polysaccharide-bioactive peptide nanoparticles can also be valuable nanocarriers for the encapsulation of small molecular polyphenols, providing better bioavailability of these valuable components (Budryn *et al.*, 2016). However, the proportions of biopolymers may have a substantial effect on the properties of the particles. For instance, Akbas *et al.* (2017) suggested that whey protein isolate outnumbered maltodextrin by one to two, which could provide higher antioxidant activities and phenolic contents with high encapsulation efficiency and excellent stability in gastric juices or at high temperatures. The mixtures of maltodextrin : whey protein isolate (50:50) were also used as wall materials in a previous study (Tsali & Goula, 2018). The results showed that proteins are an exciting alternative to the commonly used maltodextrin (Moreno, Cocero, & Rodríguez-Rojo, 2018).

2.3.3.6 Practical application of microencapsulation

Each phenolic extract or concentrate has its own profile of phenolic compounds, and the effects of pH, salinity, and organic loads are important to consider in relation to the functionality of their products. Therefore, the effects of the extract or concentrate when in

combination with different protein needs to be studied separately (Paulo & Santos, 2020). Most studies focus on the direct use of protein to bind concentrate or extract and its corresponding particle properties. The next step is how to apply these functionalised protein particles in specific formula foods to improve their colour profiles, increase the protein content, and enhance the health characteristics of food.

2.4 Application of dietary phenolics loaded protein ingredients

Only a few studies have investigated the specific application of functional capsules of phenolics and proteins. The particular application of encapsulated polyphenols depends on several factors such as particle size, food matrix, and physicochemical characteristics (Fang & Bhandari, 2010), resulting in enhanced functional properties of food, anti-microbial properties, and health-promoting properties (Milincic *et al.*, 2019). Akbas *et al.* (2017) studied the effects of the processing temperature on particles that encapsulated wheatgrass juice powder and reported that good stability against thermal treatment by releasing most of their constituents in the intestinal juice. Stanciuc *et al.* (2017) examined the thermal stability of encapsulated anthocyanins at three different temperatures (80 °C, 90 °C, and 100 °C) for 30 min. Good thermal stability of anthocyanins in both powders has been observed, which would be helpful to the industrial application of grape anthocyanins. Akdeniz *et al.* (2018) obtained onion skin extract encapsulates with excellent thermal stability which could be applied to baked and fried foods.

2.5 Storage properties

External factors (light, thermal and oxygen) are potential detrimental conditions for the qualities of capsules or capsule applied products. Moreno *et al.* (2018) concluded that the stability of antioxidant capacities of the particles depended more on storage time rather than temperature. Moreover, exposure products to normal ambient light conditions during storage

has been shown to have no significant effect on the stability of total phenolics and anthocyanins, or antioxidant activities. Aprodu *et al.* (2019) reported that spray-dried black rice extract encapsulates showed stability for thermal treatment. Calva-Estrada, Lugo-Cervantes, and Jimenez-Fernandez (2019) gave a similar conclusion with cocoa liquor whey protein encapsulates, which indicated the feasibility of using the powder in functional food. In addition, no significant influence on the physiochemical and texture properties of functional yogurt with the addition of apple peel extract encapsulate during shelf-life time (El-Messery *et al.*, 2019).

2.6 Digestion properties

Functionalised protein ingredients might have different behaviour when passing through the GI tract because of protein-phenolic interaction, resulting in masked nutrition quality and bioavailability. Encapsulated wheatgrass juice powder showed good stability against gastric juices, whereas they released most of their constituents in the intestinal juice (Akbas *et al.*, 2017). Food-protein nanoparticles are the most commonly used nanocarriers for the delivery of dietary phenolics since there is solid evidence that they improve the intestinal absorption of phenolic compounds (Hu, Liu, Zhang, & Zeng, 2017). A binary mixture of wall materials, such as polysaccharide-protein complexes coacervate is a novel strategy protecting the capsules pass through gastric digestion. Active components bound to the protein part of the nanocarrier via hydrogen bonding and hydrophobic interactions, while polysaccharides contributed to the prevention of enzymatic protein degradation in gastric conditions (Jakobek, 2015).

2.7 Toxicological considerations

Nanosized particles are supposed to have better performance in terms of encapsulation efficiency. However, these have a potential health risks due to their nanosizes (Kasaai, 2015).

Researches on gold nanoparticles have demonstrated that the nanotoxicity of the materials depends mostly on their particle sizes (Kim *et al.*, 2012). On the other hand, polyphenol toxicity is a concern when it is used as a food supplement (Jain, Manghani, Kohli, Nigam, & Rani, 2013). Hence, the toxicity of dietary phenolics loaded whey protein particles, especially in nanosize forms needed to be considered. Primary studies on nanoparticle toxicity are related to their use in medicine and pharmacology, while risk assessment about food consumption has not been investigated systematically (Milincic *et al.*, 2019).

2.8 Conclusions and trends

Each polyphenol has its unique properties, such as the binding affinities to milk proteins. Therefore, the functional proteins ingredients produced under different conditions differ from application characteristics, which need to be investigated individually. At present, the studies of the interactions of milk protein with dietary phenolics are normally based on the modification of protein structures, polymerisation of polyphenols, enzymatic treatment of protein and creation of a covalent bond between molecules. Further incorporation of these functional ingredients to food matrices as colourants, antioxidants, protein enhancers and substitutes for fats and carbohydrates will be a promising food innovation method. Besides, the toxicological data of novel polyphenols functionalised protein raw materials and their nutritional properties need to be further investigated for the purpose of safe and proper application.

Chapter 3

Materials and methods

3.1 Preparation of encapsulated materials

3.1.1 Preparation of imitation blackcurrant concentrate

Imitation blackcurrant juice, without blackcurrant polyphenols, was prepared to determine the effects of juice acids and sugars on the final powder properties (Schneider *et al.*, 2016). The buffering capacity was imitated by citric acid (0.67 M) and citrate (0.67 M). The sugar content was imitated by the addition of fructose (2.6 %) and glucose (3.9 %) (Schneider *et al.*, 2016). Citric acid (Hansells, New Zealand) and citrate (Hansells, New Zealand) were purchased from the local supermarket (New World, Christchurch, New Zealand). Glucose, fructose, and sucrose were purchased online (AnalaR NORMAPUR, VWR, Belgium). Deionised water was used to dissolve the ingredients according to the formula in Table 3.1. The pH value was stable at 2.95 ± 0.5.

Table 3.1 Formulation of the imitation blackcurrant juice

Buffer		Sugar (g/kg)			pH
Acid (citric acid)	Base (citrate)	Glucose	Fructose	Sucrose	
4.41 M	4.41 M	462.57	653.04	350.67	2.9 ± 0.5

3.1.2 Preparation of whey protein isolate-based blackcurrant encapsulate

Figure 3.1 illustrates the steps used to construct the whey protein isolate-based blackcurrant concentrate encapsulates. Whey protein isolate (WPI 895-1) (Fonterra Co-operative Group Limited, Auckland, New Zealand) was reconstituted in deionised water to make whey protein solution (10%, w/w). The solution was then kept at 4 °C for overnight to facilitate fully rehydration. Then the blackcurrant concentrate (ViBERi, Nelson, New Zealand Limited) was added into the solution in a dropwise manner until the solution pH value was stable at 4.5 ±

0.1. Similarly, imitation blackcurrant juice was used to make controls by following the same procedures. The combined solutions were processed by spray-drier (Nire Mobile MinorTM 2000, GEA, Germany) and freeze-drier (Cuddon FD 5.5, Cuddon Freeze Dry, New Zealand) immediately, and the products obtained were labelled as follows: SWB (spray-drying + whey protein isolate+ blackcurrant concentrate), SWC (spray-drying + whey protein isolate + imitation blackcurrant juice), FWB (freeze-drying + whey protein isolate + blackcurrant concentrate), and FWC (freeze-drying + whey protein isolate+ imitation blackcurrant juice). All the ingredients were stored at -20 °C for further analysis within 6 months.

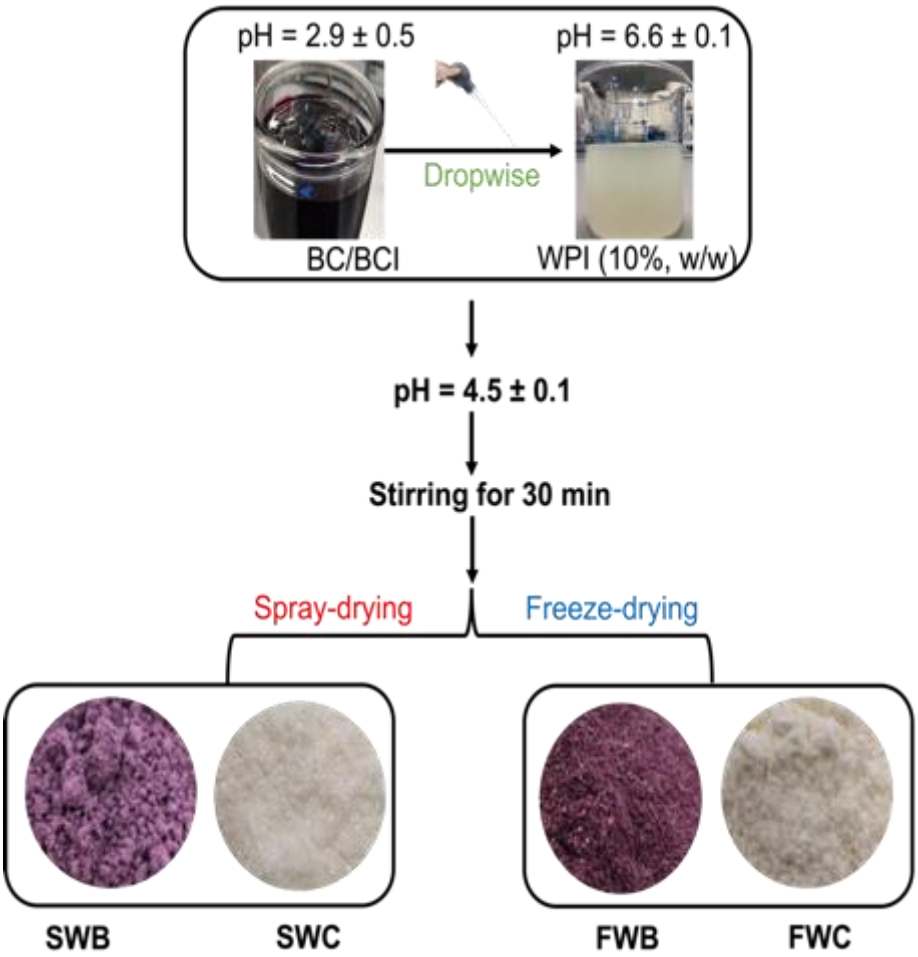

Figure 3.1 The manufacturing steps for whey protein isolate-based blackcurrant encapsulates

SWB: spray-drying + whey protein isolate+ blackcurrant concentrate; SWC: spray-drying + whey protein isolate + imitation blackcurrant juice; FWB: freeze-drying + whey protein isolate + blackcurrant concentrate; FWC: freeze-drying + whey protein isolate+ imitation blackcurrant juice).

3.1.3 Preparation of sodium caseinate-based blackcurrant encapsulate

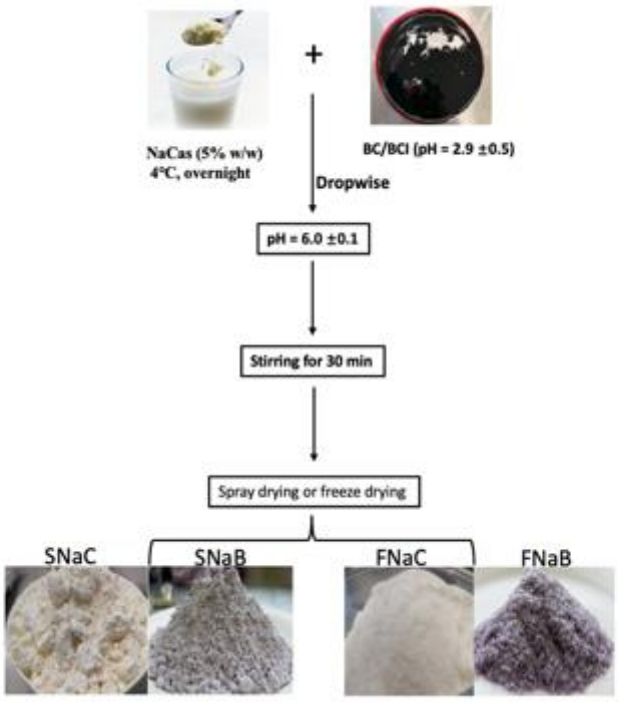

Figure 3.2 The production procedures for sodium caseinate (NaCas)-based blackcurrant concentrate encapsulates.

SNaB: spray-drying + sodium caseinate + blackcurrant concentrate; SNaC: spray-drying + sodium caseinate + imitation blackcurrant juice; FNaB: freeze-drying + sodium caseinate + blackcurrant concentrate; FNaC: freeze-drying + sodium caseinate+ imitation blackcurrant juice)

Figure 3.2 illustrates the preparation procedures of sodium caseinate-based encapsulate. Sodium caseinate (Fonterra Co-operative Group Limited, Auckland, New Zealand) was dissolved in deionised water to make a protein solution (5 g/100 g). The solution was then kept at 4 °C overnight to facilitate rehydration. The blackcurrant concentrate (ViBERi, Nelson, New Zealand Limited) was added to the solution in a dropwise manner until the solution pH value was stable at 6.0 ± 0.1. The solutions were processed immediately by spray-drying (Nire Mobile MinorTM 2,000, GEA, Germany) (inlet temperature = 120 ± 5 °C; outlet temperature = 60 ± 5 °C), and freeze-drying (Cuddon FD 5.5, Cuddon Freeze Dry, New Zealand) (-40 °C for 24 h), respectively. The procedures were followed with the addition of imitation blackcurrant juice to produce controls. The obtained powders were labelled as follows: SNaB (spray-drying

+ sodium caseinate +blackcurrant concentrate), SNaC (spray-drying + sodium caseinate + imitation blackcurrant juice), FNaB (freeze-drying + sodium caseinate +blackcurrant concentrate), and FNaC (freeze-drying + sodium caseinate + imitation blackcurrant juice). All of the powders were stored at -20 ℃ for further analysis.

3.2 Cookie production

The AACC method (10-50D) (AACC, 1995) was used for the production of functional cookies with slight modifications. All ingredients, 64 g of shortening (Kremelta, Peerless foods, Sydney, Australia), 150 g of sugar (Cane white sugar, Auckland, NZ), 2.5 g of soda (Hansells Baking Ltd., Australia), 2.1 g of salt (iodised salt, Pams Products Ltd., Auckland, New Zealand) were mixed in an electric mixer (Breville the linetix wize, BFP 450, NSW, AU) at speed 1 for 3 min before adding 50 g of deionised water, and then immediately turned to speed 2 for 1 min. The whole meal wheat flours (Champion Flour, Auckland, New Zealand) and functional protein ingredients were added and mixed at speed 2 for two more minutes, meanwhile scraping down at every 30 s. The specific information on the formula for each cookie at different replacement level was described in Table 3.2. The dough was rolled to a thickness of 6 mm by roller with an aluminium guide. Then the dough was cut with a cookie cutter of 57 mm in diameter. The cookies were placed on metal trays and baked in an electric oven at 180 °C (BAKBAR turbofan convection oven, E311, Moffat Pty Ltd., Sydney, AU) for 9 min. Cookies were cooled to room temperature before being wrapped in foil, and then placed in airtight polyethene bags, and stored at -20 °C for further analysis.

3.3 Moisture content of encapsualtes and cookies

The moisture content of samples, including encapsulates and cookies, were measured according to the method as described by Patil *et al.* (2018). The sample (2.00 g) was weighed into a dry crucible (pre-weighed) on an analytical balance (ARC120; OHAUS Corp., Parsippany,

NJ, USA). Then, the crucible was placed into an oven (105 °C, overnight) before it was put into a desiccator immediately for an hour to let the sample cool down to room temperature (22 °C). The dry empty crucible weight was recorded as W1. The sample weight was recorded as W2. The crucible weight with drying sample was recorded as W3. The results were calculated as the equation (3-1) below:

$$\text{Moisture content } (\%) = \frac{\text{W1} + \text{W2} - \text{W3}}{\text{W2}} \times 100\% \quad (3\text{-}1)$$

Table 3.2 Amount of experimental ingredients

Sample	Flour (g)	Functional powder (g)	Shortening (g)	Sugar (g)	Salt (g)	Sodium bicarbonate (g)
0%	225.00	0.00	64.0	150.0	2.1	2.5
5%	213.75	11.25				
10%	202.50	22.50				
15%	191.25	33.75				

3.4 Ash content encapsualtes and cookies

The ash content of samples, including encapsulates and cookies, was measured according to the method as described by Patil *et al.* (2018). The crucible (W3) from section 3.2 was put into a furnace (5 h, at 550 °C) (Mufla, INDEF). The sample were cooled to room temperature by putting into a desiccator for an hour. The crucible with ash was recorded as W4. The ash content of the sample was calculated as equation (3-2) below:

$$\text{Ash content } (\%, \text{dry basis}) = \frac{\text{W4} - \text{W1}}{\text{W3}} \times 100\% \quad (3\text{-}2)$$

3.5 Protein content and C/N of encapsualtes and cookies

The total carbon and nitrogen content of encapsulates and cookies were analysed using Elementar Vario Max CN Elemental analyzer (Elementar GmbH, Hanau, Germany). The sample was combusted at 900 °C in an oxygen atmosphere. The combustion process converted any elemental carbon and nitrogen into CO_2, N_2 and NO_x. The NO_x species were subsequently

reduced to N_2. These gases were then passed through a thermal conductivity cell to determine CO_2 and N_2 concentrations and the percentage of C and N were calculated from the sample weights. The conversion factors we used was 6.28 for protein content.

3.6 Colour profile of encapsualtes, cookie dough, and cookies

The colour of samples, including encapsulates, cookie dough, and cookie products, was measured using a colourimeter CR-210 (Minolta, Tokyo, Japan). Lightness (L^*), redness (a^*), and yellowness (b^*) values were recorded to calculate the total colour difference ΔE between each sample and corresponding controls according to the equation below (Michalska, Wojdyło, Lech, Łysiak, & Figiel, 2017)

$$\triangle E = \sqrt{({L_{Sample}}^* - {L_{Control}}^*)^2 + ({a_{Sample}}^* - {a_{Control}}^*)^2 + ({b_{Sample}}^* - {b_{Control}}^*)^2} \quad (3\text{-}3)$$

3.7 Chemical extraction

All the samples, including protein encapsulates and cookies, were extracted according to the method described by Floegel, Kim, Chung, Koo, and Chun (2011). The sample powder (2.0 g) was extracted with 20 mL of 70% methanol (25 °C, 12 h). Afterwards, the slurry was treated with ultrasonication (4 °C water bath, 30 min, 100 Hz), then centrifugation (5,000 × g, 20 min) and finally, rotary evaporation (40 °C). The final volume of the extract was adjusted to 20 mL with deionised water and stored at -20 °C for analysis.

3.8 *In vitro* digestion and glycaemic glucose equivalent assay

An *in vitro* digestion including gastric and intestinal steps was performed as described by Gao, Brennan, Mason, and Brennan (2016). The entire procedure was performed in 37 °C. Two g of each lyophilised sample was added to 20 mL of distilled water and the pH value of the mixture was adjusted to 2.0 with 6 mol/L HCl. For the gastric digestion, pepsin (Sigma-Aldrich, St. Louis, MO, USA) was added at a concentration of 0.05 g/mL of sample. After incubating for 30 min

(37 °C), a 1 mL of aliquot from each sample was taken (time 0) and added to 4 mL of absolute ethanol individually to quench the reaction. For the intestinal step, the pH of the digest was adjusted to 6.0 by the dropwise addition of 0.9 M $NaHCO_3$. After the pH adjustment, 0.1 mL of α-amyloglucosidase (3000 U/mL) was added. The digestion time began as soon as 5 mL of pancreatin-bile solution (3 g/mL pancreatin and 0.025 g/mL bile salts in 0.1 M $NaHCO_3$, pH = 7.4) was added. At 20, 60 and 120 min, 1mL of aliquots from each sample were taken and treated with ethanol as above individually. All of the aliquots were centrifuged at 2,500 × g for 20 min and the supernatants were collected and then filtered through 0.45 µm filter and stored in microtubes at -20 °C for analysis.

The glycaemic glucose equivalent assay was carried out by evaluating the amount of reducing sugar released over a period of 120 min (Monro, Mishra, & Venn, 2010). The 3.5-dinitrosalicylic acid method was carried out to evaluate the amount of reducing sugar released during the *in vitro* digestion. The amount of reducing sugar released in mg glucose/g sample was calculated and plotted versus time, while the area under the curve was calculated by dividing the graph into trapezoids.

3.9 High performance liquid chromatography

The main anthocyanin categories and contents of the particles were identified by high performance liquid chromatography using an Agilent 1200 series high performance liquid chromatography (Agilent Technologies, Santa Clara, CA, USA) equipped with a photodiode array detector. Samples were extracted according to the method by Floegel *et al.* (2011). The sample extracts obtained were then removed glucosides under the condition of 2 mol/L HCl, followed by boiling water bath treatment for 1 h. The samples were then filtered through a 0.2 mm PTFE syringe filter (Fisher Scientific, Fair Lawn, NJ, USA) and analysis was performed using a C18 column (4.6 x 250 mm, 5 µm) (Supelco, Bellefonte, PA, USA). The column was

operated at a temperature of 30 °C. A linear gradient from 5% A to 60% B in 30 min was used for the high performance liquid chromatography assay. Solvent A was acetonitrile and solvent B was water containing 10% formic acid. The flow rate was 0.8 mL/min and the absorption spectrum was recorded at 520 nm (Yan, Zhang, Zhang, & Zheng, 2016). Two replicates were performed in the analysis.

3.10 Total phenolic content

Total phenolic content of the sample extract (from extraction step as described in 3.6) and sample digesta (from extraction step as described in 3.7) were measured by the method described by Archaina, Leiva, Salvatori, and Schebor (2018). The samples, including encapsulates and cookies, were analysed. Gallic acid was used as a standard to make the calibration carve. The results were expressed as mg gallic acid equivalents per gram sample (µg gallic aid equivalent/g). Standard solutions of gallic acid ware prepared at 0, 12.5, 25, 50, 75, 100, and 150 µg/mL in 70% methanol. Each standard (0.5 mL) or sample extract (0.5 mL) was placed in tubes, followed by adding 2.5 mL of 0.2 mol/L Folin-Ciocalteu reagent and 2 mL of 7.5m% sodium carbonate, and kept in a water bath (Thermo Scientific Precision GP 28) at 40 °C for 30 min. The samples were allowed to cool to room temperature (22 °C) for at least 5 min prior to reading absorbances by a spectrophotometer at 760 nm.

3.11 Total anthocyanin content and encapsulation efficacy

The encapsulates were processed according to the method described by Condurache *et al.* (2019). For the total anthocyanins, 100 mg of encapsulates was mixed with 1 mL ethanol, acetic acid and water (50:8:42) on vortex for 1 min, then centrifuged at 2000 g for 3 min. The liquid part was collected and filtered through a 0.45 µm filter. Similarly, for surface anthocyanins, 100 mg of sample was dispersed in 1 mL of ethanol and methanol (1:1) mixture.

The mixture was vortexed for 1 min, centrifuged for 2 min, and the supernatant was separated.

The pH-differential method was carried out to quantify the total anthocyanin content (TAC) and surface anthocyanin content (SAC) of the supernatants. Encapsulation efficacy (EE) was calculated based on the equation (3-4) below:

$$\%\ \text{Encapsulation Efficacy (EE)} = \frac{\text{TAC} - \text{SAC}}{\text{TAC}} \times 100\% \qquad (3\text{-}4)$$

3.12 Antioxidant activity of encapsulates and cookies

3.12.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The free radical scavenging ability of extract and digesta was qualified by DPPH assay according to the method described by Trigueros, Wojdyło, and Sendra (2014) with slightly modifications. A volume of 0.5 mL of the sample was added to 1 mL of freshly prepared 0.1 mmol/L DPPH solution. The mixtures were incubated in the dark for 30 min. The absorbance at 517 nm was determined by a spectrometer. Trolox was used as a standard and the result was expressed as μmol Trolox equivalent (TE)/g sample.

3.12.2 Ferric reducing antioxidant power assay

The ferric reducing antioxidant power assay was conducted to measure the ferric reducing powder of the samples as previously described by Benzie and Strain (1996). The ferric reducing antioxidant power assay reagent solution was prepared with 300 μmol/L acetate buffer (pH = 3.6), 10 mmol/L TPTZ (dissolved in 40 mmol/L HCl) and 20 mmol/L $FeCl_3$ at a ratio of 10:1:1 (v/v/v). The ferric reducing antioxidant power assay reagent solution (2.5 mL) was mixed with 250 μL sample extract or digesta. The mixture was incubated in the dark for 2 h at 37 °C and the absorbance was measured at 593 nm. $FeSO_4$ solution was used as a standard. Results were expressed as μmol Fe^{3+} equivalent (Fe^{3+} E)/g sample.

3.13 Alpha-amylase inhibition assay of encapsulates extracts

Alpha-amylase (α-amylase) inhibition assay was conducted as described previously by Zengin, Sarikurkcu, Aktumsek, and Ceylan (2014) with some modifications. A sample of extract (25 µL) was dissolved in 0.01 M phosphate buffer solution (pH = 6.9, with 6 mM NaCl). A 50 µL aliquot of α-amylase (0.5 mg/mL) was pre-incubated in each well of a 96-well plate at 37 °C for 10 min. A blank was set by combining the sample with buffer instead of enzyme. Then 50 µL of 1% starch solution was added to each well to initiate the reaction. The reaction was stopped by adding 25 µL of 1 mol/L HCL to each well. After adding 100 µL of 1% iodine-potassium iodide indicator solution to each well, the absorbance at 630 nm was measured by a microplate reader. The results were expressed as half inhibition concentration (IC_{50}) (µg/mL).

3.14 Foamability and foam stability, and microscopic structure of encapsulates

Foam generation was performed following the method of Liu, Elmer, Low, and Nickerson (2010) with some modifications. Protein powder (2 g) was added into 20 mL of deionised water in a 50 mL of centrifuge tube. The mixture was homogenised using a Model OMNI MIXER 17106 Homogeniser (DuPont Instruments, Sorvall Omni-Mixer, United States) at a speed of 4,000 rpm for 5 min. After the homogenisation was completed, the sample was transferred to a graduated cylinder. A spatulate was used to remove any remaining foam from the beaker. Using a spatula level off the top of the foam and record the foam volume. The protein solution volume was recorded as V_0. The total volume of foam was recorded as V_p. Foamability was expressed as V_0/V_p (%). After 30 min, the volume of the foam remaining was recorded as V_r. Foaming stability was expressed as V_r/V_p (%).

The shape and size of air bubbles were observed at 0 min (within 1 min after homogenisation) and 30 min by phase contrast microscopy. The foam was layered on a glass slide with a cover slip. Images under a phase contrast mode were acquired with a MICROPHOT-FXA

photomicroscope fitted with a 4 X objective lens and equipped with a built-in digital camera (Nikon Inc., Garden City, NY, USA) (Kamath, Huppertz, Houlihan, & Deeth, 2008).

3.15 Water holding capacity and oil holding capacity of encapsulates

The method as previously described by Wasswa, Tang, Gu, and Yuan (2007) was used. Water holding capacity (WHC)/oil holding capacity (OHC) was determined by adding 1 g of protein in 9 g of water/oil in a 50 mL of screw cap centrifuge tube. Samples were vortexed for 10 s every 5 min for a total of 30 min, making sure the samples were thoroughly wetted, and then centrifuged (Hettich Rotina 380 Benchtop centrifuge, type 1701, Germany) at 4,000 × g for 15 min. The supernatant was carefully decanted, and the remaining pellet was weighed. The water/oil holding capacity was calculated as the wet sample weight minus the dry sample weight, divided by the dry sample weight (3-5).

$$\text{WHC}(\%) \text{ or } \text{OHC}(\%) = \frac{\text{Wet sample weight} - \text{Dry sample weight}}{\text{Dry sample weight}} \times 100\% \qquad (3\text{-}5)$$

3.16 Scanning electron microscopy of encapsualtes

The morphology, and surface appearance, of the encapsulates were observed with a scanning electron microscopy (Evo 18, ZESS, Germany) operated at 10.00 kV acceleration voltage under a vacuum condition. A thin layer of powder sample was evenly affixed with double-sided carbon tape to an aluminium stub. The surfaces of particles were sputtered with gold to make the sample conductive, observed and photographed (Gong *et al.*, 2019).

3.17 Bulk density of encapsualtes

Bulk density of encapsulates was measured according to the methodology described by Correia, Grace, Esposito, and Lila (2017) with some modifications. Sample (2 g) was placed in a 10 mL graduated cylinder. The cylinder was tapped by hand and the bulk density was

calculated as the ratio of the mass of powder contained in the cylinder to the volume occupied.

3.18 Dynamic light scattering measurement of particles sizes and sizes distribution

The particle sizes of encapsulates were measured using laser diffraction with a Mastersizer 3000 (Malvern, Worcestershire, UK) (Kwak, Lee, Ahn, & Jeon, 2009). The powder sample was added to the menthol solvent until there was between 10% and 20% obscuration. The refractive index of methanol was set as 1.33. The measurements were repeated at least five times to ensure repeatability. The results were expressed as volume weighted mean diameter D[4,3]. The particle size distribution was represented by span factor, which is defined as

$$\text{Span} = \frac{\mathrm{D_{90}} - \mathrm{D_{10}}}{\mathrm{D_{50}}} \times 100\% \qquad (3\text{-}6)$$

where D_{90}, D_{10}, and D_{50} corresponds to the diameters at which the cumulative sample volumes were under 90%, 10%, and 50%, respectively.

3.19 Texture analysis of cookie dough and cookies

The dough and cookies have different texture profiles. The texture of dough and cookies were analysed separately by different models of texture analyser (TA. XT plus Texture Analyzer, Stable Micro Systems, Godalming, UK).

3.19.1 Dough texture analysis

The hardness of dough and cookies were measured using a texture analyser (TA. XT plus Texture Analyzer, Stable Micro Systems, Godalming, UK), separately. Measurement the hardness of cookie dough by penetrating with a cylinder probe (6 mm, P/6). The analyser was set at a load cell of 5kg; Measure Force in Compression Mode; Pre-test Speed 2.0 mm/s; Test Speed 3.0 mm/s; Trigger force 5 g.

3.19.2 Cookie texture analysis

Measurements of the hardness of cookies was carried out by a 3-point bend rig method (Hossain *et al.*, 2017). The analyser (TA. XT plus Texture Analyzer, Stable Micro Systems, Godalming, UK) was set at a load cell 50 kg; pre-test speed 2 mm/s; test speed 5 mm/s; post-test speed 10 mm/s; return to start mode. Each cookie was placed on the support ring and the probe moved downward until the samples were broken. The peak force (kg) was recorded as hardness. All measurements were made in triplicate.

3.20 Diameter, thickness, and baking loss

Cookie diameter (mm) and thickness (mm) were measured using callipers (INSIZE digital calliper, series 1112, INSIZE Inc., Loganville, GA, USA). The cookie was weighed before putting into an oven and after the baking process and recorded separately. The baking loss (%) was determined by the following formula:

$$\text{Baking loss } (\%) = \frac{\text{Weight}_{\text{Before baking}} - \text{Weight}_{\text{After baking}}}{\text{Weight}_{\text{Before baking}}} \times 100\% \qquad (3\text{-}7)$$

3.21 Cell culture

The human hepato-carcinoma cell line, HepG2, was obtained from American Type Culture Collection (Manassas, VA, USA). HepG2 was grown in DMEM culture medium supplemented with 10% heat inactivated FBS, penicillin (100 U/mL), and streptomycin sulphate (100 µg/mL) at 37 °C in a humidified atmosphere of 5% CO_2.

3.22 Cell viability

HepG2 cells were treated with sample extracts at a density of 5.0×10^3 cells/well in 96-well culture plates for 48 h. Cell viability was determined by using a Cell Counting Kit-8 (CCK-8) assay kit (Dalian Meilun Biotechnology Co., Ltd, Dalian, China) according to the instructions of the manufacturer. Absorbance was calculated for all samples at 450 nm (OD450). The relative

cell viability was presented after normalised to untreated cells (control). Cell viability rates were measured in 24 h and were calculated based on OD450 value. Cell viability rate (%) = A450 (test)/A450 (control) × 100%.

3.22.1 Cell apoptosis

The cells were plated at a density of 3.0×10^5 cells/well in a 6-well plate and incubated overnight in an incubator. The growth media was used as control. After treating with different concentrations of sample extracts individually for 24 h, the cells were washed twice with phosphate buffer solution. Cell apoptosis was analysed using an Annexin V-FITC/PI Apoptosis Detection Kit (V13241) (Thermo, MA, USA) according to the manufacturer's instructions. Briefly, 5 µL of Annexin V-FITC and 1 µL of 100 µg/mL PI were added followed by the incubation of the cells in the dark for 15 min. The apoptotic rate was examined using a fluorescence-activated cell sorting flow cytometer. After staining, all samples were immediately measured on a CytExpert flow cytometer (CytoFLEX S, Beckman Coulter, USA). CytExpert Software (CytoFLEX S, Beckman Coulter, USA) was applied to analyse data.

3.22.2 Reactive oxygen species generation

The intracellular production of reactive oxygen species was assessed using an oxidant-sensitive fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate with a flow cytometer (Kitagawa *et al.*, 2015). Cells were seeded in 100-mm culture dishes, and then treated with 200 µg/mL sample solution in the dark. The cells were incubated for 24 h after exposure to the sample. The cells were then incubated with 10 µM 2',7'-dichlorodihydrofluorescein diacetate for 15 min at 37 °C and washed twice with phosphate buffer solution. Then, 2',7'-dichlorodihydrofluorescein diacetate fluorescence was analysed using a flow cytometer (excitation, 488 nm; emission, 525/50 nm band-pass filter). All procedures were conducted in

the dark to avoid photoactivation of 2',7'-dichlorodihydrofluorescein diacetate. Analyses of flow cytometric data were carried out using FlowJo (version 7.2.2, Tree Star, Ashland OR).

3.23 Molecular docking

The *in silico* approach was used to investigate the interaction between α-amylase and the four major anthocyanins found in blackcurrant, namely 3-O-rutinoside (D3R), delphinidin 3-O-glucoside (D3G), cyanidin 3-O-glucoside (C3G), and cyanidin 3-O-rutinoside (C3R). The 3D structure of D3G (CAS:6906-38-3), D3R (15674-58-5), C3G (7084-24-4), C3R (28338-59-2) were downloaded from PubChem and further processed by Chembio3D. The key target protein (1MFV) was downloaded from the Protein Data Bank database (Homoki *et al.*, 2016). The protein was uploaded into PyMoL (2.3.0) to remove the original ligand and water molecules. The protein was then imported into AutoDocktools (V1.5.6) for hydrogenation, charge calculation, charge distribution, atom type designation. PyMOL and Ligplot were used to analyse the interaction mode of docking results.

3.24 Statistical analysis

All the determinations were carried out in triplicate unless otherwise stated. Results were presented as mean ± standard deviation. One-way analysis of variance (ANOVA) was employed to do statistical analysis and significant differences were determined by Tukey's multiple comparison test ($p < 0.05$ or $p < 0.01$). Where applicable, a two-way analysis, using GraphPad Prism software (version 8.0, GraphPad Software, Inc. San Diego, USA), was conducted for data analysis. Pearson's correlation was conducted using GraphPad Prism software version 8.0 (version 8.0, GraphPad Software, Inc. San Diego, USA).

Chapter 4

Fortification of whey protein isolate with blackcurrant juice concentrate

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4.1 Abstract

A whey protein isolate aqueous solution was combined with blackcurrant juice concentrate via spray- and freeze-drying techniques separately. Chemical compositions (ash, moisture, protein, carbohydrate, total phenolic content, and total anthocyanin content), antioxidant activity, anthocyanin encapsulation efficiency, predictive *in vitro* glycaemic response, and α-amylase inhibitory activity were analysed. The intestinal digesta, following an *in vitro* digestion, was collected to investigate the alteration of total phenolic content, and antioxidant activity of the protein ingredient. A molecular docking study was carried out to reveal the potential binding actions between the four main blackcurrant anthocyanins and α-amylase. The results showed that both spray-drying and freeze-drying encapsulates could be used as natural colourants, protein enhancers, antioxidants, and postprandial blood glucose regulators. Spray-dried particles presented a higher ($p < 0.05$) total phenolic content and total anthocyanin content, and higher encapsulation efficacy (99.64 ± 0.16 %), compared with freeze-drying particles. Thus, blackcurrant juice concentrate, fortified whey protein isolate particles through spray-drying strategy, might be a good option for the food industry to produce functional protein ingredient.

4.2 Graphic abstract

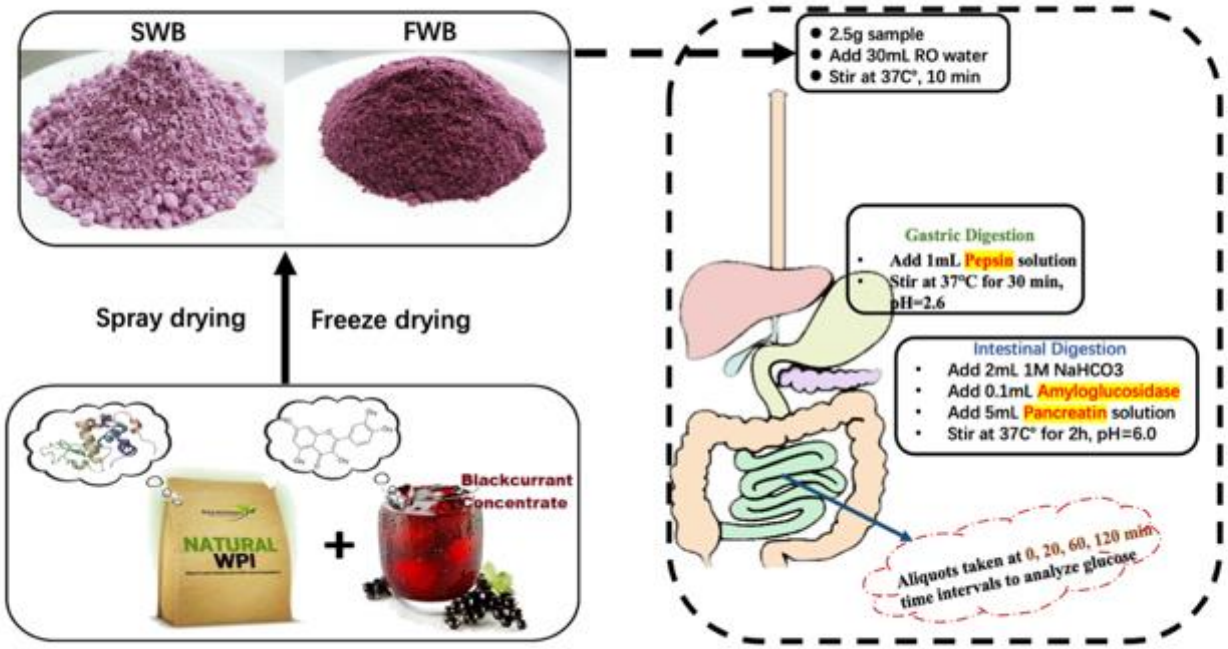

Figure 4.1 Sample preparation process and *in vitro* digestion conditions

4.3 Introduction

Whey, a cheese production by-product, is processed into valuable protein ingredients, which are used in formula foods due to their functional and nutritional properties, such as foaming ability, emulsion capacity, gelling properties, high solubility, rapid digestibility, and extensive amino acid profile (Kilara & Vaghela, 2018). There has been a steady increase in the production of whey protein each year worldwide as cheese and milk powder use has also increased (Lagrange, Whitsett, & Burris, 2015). Hence, the valorisation of this by-product is of vital importance (Tsali & Goula, 2018; Zhan, Shi, Wang, Li, & Chen, 2019). The most common commercial form of whey protein is whey protein isolate with ≥ 90% protein content (Bansal & Bhandari, 2016). Proteins have been utilised as carrier agents for sensitive bioactive components, and whey protein isolate is one of the most widely studied material due to its

high acceptance, complete amino acid profile, widely availability and application in the food sector (Tontul & Topuz, 2017).

As a commonly consumed berry fruit, blackcurrant has numerous phytochemical and is exceptionally rich in vitamin C and anthocyanin content, which provides potent antioxidant properties, and can be used as natural colourant owning to its colouring matters, such as delphinidin 3-O-rutinoside, delphinidin 3-O-glucoside, cyanidin 3-O-glucoside, and cyanidin 3-O-rutinoside, which collectively represent approximately 90% of the total anthocyanin content (Bishayee *et al.*, 2010; Cortez & Gonzalez de Mejia, 2019). Epidemiological studies have shown that dietary intake of blackcurrant could modulate type 2 diabetes mellitus (Guo, Yang, Tan, Jiang, & Li, 2016; Iizuka, Ozeki, Tani, & Tsuda, 2018). Castro-Acosta *et al.* (2016) reported that drinks containing blackcurrant extracts could decrease postprandial blood glucose, insulin and incretin content. Commercially produced blackcurrant is mainly processed into juice concentrate with extreme acidic pH value (~2.9), which is not suitable for direct consumption (Jaworska, Sady, Grega, Bernas, & Pogon, 2011). Besides, bioactive compounds in blackcurrant concentrate are unstable during storage since these compounds are influenced by their surrounding conditions (temperature, light, oxygen) (Hellstrom, Mattila, & Karjalainen, 2013). Thus, the combination of blackcurrant concentrate with whey protein isolate might be a promising strategy to protect and delivery the functionality of blackcurrant berry fruit.

Spray-drying and freeze-drying are common methods to remove water content from products and are frequently chosen as encapsulation strategies. Spray-drying is up scalable and economic feasible, but operated under high temperature conditions (Papoutsis *et al.*, 2018), and the fact that high temperatures are reached is not beneficial for the preservation of thermolabile components. Freeze-drying is a time-consuming and costly method, but under

mild temperature condition (Rezvankhah, Emam-Djomeh, & Askari, 2020), which, in contrast to spray-drying, is good for the preservation of thermal sensitive bioactive components. Bioactive components, such as anthocyanins, are known to be highly thermal sensitive. The specific effects of different drying methods on blackcurrant concentrate-whey protein isolate particles are still not clear. In addition, the combination and interaction of blackcurrant concentrate with protein needs to be investigated. Previous studies have evaluated the encapsulation of grape pomace extract (Moreno *et al.*, 2018), black rice aqueous anthocyanin-rich extract (Aprodu *et al.*, 2019), and lotus seedpod proanthocyanins (Chen, Zhang, Xie, Sun, & McClements, 2020) with whey protein isolate. However, there are few studies conducted in a food-compatible manner to produce encapsulates. This paucity of information limits the utilisation of these ingredients into functional food products. The formation of protein-polyphenol particles in powder form can broaden the application of both proteins and polyphenols in food system (Foegeding *et al.*, 2017). Therefore, in this study, we utilised the low pH value of blackcurrant concentrate, and the solubility of whey protein isolate in a wide acidic pH range, to neutralise the solutions to the isoelectric point (pH = 4.5) of whey protein, resulting in the formation of larger particles. This may result in a degree of embedding and protection for sensitive components of blackcurrant concentrate. Physicochemical properties, antioxidant activity, and glycaemic effect of the functionalised particles was investigated. Simultaneously, the efficiency of drying techniques was also compared.

4.4 Methods

4.4.1 Preparation of protein ingredients

The production procedures of the novel protein ingredients (SWC, SWB, FWC, FWB) were following the steps as described in 3.1.

4.4.2 Moisture content measurement

The moisture content of the encapsulates was measured as described in 3.3.

4.4.3 Ash content measurement

The ash content was carried out as described in 3.4.

4.4.4 Total carbon and protein content

The total carbon and protein content was measured as described in 3.5.

4.4.5 Colour measurement

The colour profiles of the samples were recorded by following the methods described in 3.6.

4.4.6 Chemical extraction

The chemical extraction was performed as described in 3.7.

4.4.7 Simulation of the *in vitro* digestion process and glycaemic glucose equivalent

The simulation of the *in vitro* digestion process and glycaemic glucose equivalent assay was carried out as described in 3.8.

4.4.8 Determination of total phenolic content

The total phenolic content was determined as described in 3.10.

4.4.9 Determination of total anthocyanin content, surface anthocyanin content, and encapsulation efficiency

The total anthocyanin content, surface anthocyanin content, and encapsulation efficiency were determined as described in 3.11.

4.4.10 Antioxidant activity

The antioxidant activity measurement was carried out as described in 3.12.

4.4.11 Alpha-amylase inhibition assay

Alpha-amylase inhibition assay was conducted as described in 3.13.

4.4.12 Molecular docking study

Molecular docking studies was performed as described 3.23.

4.4.13 Statistical analysis

Statistical analysis was carried out as described in 3.24.

4.5 Results and discussion

4.5.1 Chemical composition analysis and colour profiles

Chemical components of all the samples including ash, water, protein and carbohydrate/protein ratio (C/N) are listed in Table 4.1. The addition of blackcurrant concentrate increased the ash content ($p < 0.05$), whereas decreased the protein content and C/N value ($p < 0.05$), owing to the extra sugar and ash content of blackcurrant concentrate. There was no significant difference among all samples for the moisture content under the defined conditions. Both SWB and FWB (with about 68 % protein), have the potential to be utilised as novel high protein food additives to replace carbohydrate and/or fat in food. Tumbas Šaponjac *et al.* (2016) incorporated whey-protein-sour cherry encapsulates in cookie

product to create functional snacks with lower sugar content, higher protein content, and extra health benefits from sour cherry.

Table 4.1 Chemical composition (%) analysis of the encapsulates

	Ash (%)	Moisture (%)	Protein (%)	C/N
SWC	1.48 ± 0.09[b]	6.32 ± 0.54[a]	76.91± 0.62[a]	4.84 ± 0.02[a]
SWB	2.85 ± 0.07[a]	6.96 ± 0.35[a]	67.94 ± 0.47[b]	4.37 ± 0.03[c]
FWC	1.64 ± 0.01[b]	5.99 ± 0.03[a]	75.90 ± 0.36[a]	4.65 ± 0.05[b]
FWB	2.84 ± 0.05[a]	6.16 ± 0.44[a]	68.16 ± 0.77[b]	4.38 ± 0.01[c]

Means ± standard deviation (n = 3). Values in the same column with different letters differ significantly (p < 0.05). SWB: spray-dried whey protein isolate + blackcurrant concentrate; SWC: spray-dried whey protein isolate + imitation blackcurrant juice; FWB: freeze-dried whey protein isolate + blackcurrant concentrate; FWC: freeze-dried whey protein isolate+ imitation blackcurrant juice. All measurements were based on dry basis.

Colour parameters are the most direct indicators for food products containing anthocyanins, relating to quality and acceptance (Patras, Brunton, O'Donnell, & Tiwari, 2010; Saponjac *et al.*, 2016). For the protein ingredients, the colour parameters are mainly influenced by the amount of blackcurrant concentrate added, as well as other factors such as processing temperature, light, and oxygen (Goncalves *et al.*, 2007). Table 4.2 shows the *L**, *a**, *b**, and *ΔE* values for all protein ingredients. All samples were significantly different with their controls in terms of *L**, *a**, *b**, and *ΔE,* excepting for the *ΔE* values of SWB and FWB. These differences could be attributed to different drying strategies and the addition of blackcurrant concentrate. It is noticeable that *ΔE* (22.07 ± 0.51) of FWB is slightly higher than *ΔE* (20.52 ± 0.13) of SWB. It might be caused by the difference of surface anthocyanins content or the difference of the particle's morphology. There is a significant difference observed in *a** value, indicating that both SWB and FWB are potential natural colourants.

Table 4.2 Colour profile of the encapsulates

Sample	*L**	*a**	*b**	*⊿E*
SWC	96.89 ± 0.19[a]	-0.73 ± 0.05[a]	4.88 ± 0.22[a]	51.68 ± 0.10[a]
SWB	52.81 ± 0.50[c]	22.80 ± 0.09[b]	-10.58 ± 0.0.12[b]	20.52 ± 0.13[b]
FWC	90.41 ± 0.12[b]	-1.69 ± 0.04[c]	9.41 ± 0.42[c]	46.05 ± 0.04[c]
FWB	26.47 ± 0.51[d]	13.70 ± 0.0.08[d]	-7.02 ± 0.12[d]	22.07 ± 0.51d

Mean ± standard deviation. Means with different letters within the same column are statistically different ($p < 0.05$). SWB: spray-dried whey protein isolate + blackcurrant concentrate; SWC: spray-dried whey protein isolate + imitation blackcurrant juice; FWB: freeze-dried whey protein isolate + blackcurrant concentrate; FWC: freeze-dried whey protein isolate+ imitation blackcurrant juice

4.5.2 Total anthocyanin content, surface anthocyanin content, and encapsulation efficacy

Table 4.3 illustrates that the spray-drying treatment retained more total anthocyanin content, but less surface anthocyanins content, leading to higher encapsulation efficiency (%). These results are in agreement with the discussion in section 4.6.4. namely that spray-drying was useful in retaining high levels of phenolics. Freeze-drying is based on the removal of water from the frozen product by sublimation. It has been used as an alternative method for encapsulation of anthocyanins since anthocyanins can be absorbed on the surface of protein molecules during the freeze-drying process due to its water solubility (Ezhilarasi, Indrani, Jena, & Anandharamakrishnan, 2013). Therefore, freeze-dried particles were found to have more surface anthocyanins content, which is exposed to oxygen, especially when grinding the lyophilised material (Papoutsis *et al.*, 2018), resulting in the oxidation of anthocyanins. The spray-drying process involves the atomisation of the feed solution into a spray of very small sizes using a two-fluid atomiser powered by a compressed air supply. This process is conducive to the protein particles to wrap small molecules in the interior to form an envelope, thus having a protective effect (Papoutsis *et al.*, 2018).

Table 4.3 Encapsulation efficiency (EE%) of blackcurrant concentrate anthocyanins by spray-drying and freeze-drying

Anthocyanins	TAC (μg/100 g)		SAC (μg/100 g)		EE %	
	SWB	FWB	SWB	FWB	SWB	FWB
D3G	13372.69 ± 219.96	10298.79 ± 156.97***	36.24 ± 2.69	611.52 ± 27.82***		
D3R	9935.65 ± 101.09	7405.00 ± 151.72***	26.88 ± 1.43	444.61 ± 20.05***		
C3G	5258.54 ± 107.18	3778.45 ± 123.15***	18.93 ± 1.53	283.38 ± 16.58***	99.64 ± 0.16	95.43 ± 0.14***
C3R	56823.92 ± 162.27	42748.00 ± 203.69***	147.15 ± 6.18	2558.91 ± 113.12***		
TAC	*85390.80 ± 162.81*	*64230.24 ± 441.08****	*229.20 ± 11.83*	*3898.41 ± 121.67****		

*Means ± standard deviations (n = 3). Significant difference between spray-drying and freeze-drying is indicated by the asterisk (*p < 0.05, **p < 0.01, ***p < 0.001). TAC: total anthocyanin content; SAC: surface anthocyanin content; D3R: Delphinidin 3-O-rutinoside; D3G: Delphinidin 3-O-glucoside; C3G: Cyanidin 3-O-glucoside; C3R: Cyanidin 3-O-rutinoside. SWB: spray-dried whey protein isolate + blackcurrant concentrate; SWC: spray-dried whey protein isolate + imitation blackcurrant juice; FWB: freeze-dried whey protein isolate + blackcurrant concentrate; FWC: freeze-dried whey protein isolate+ imitation blackcurrant juice*

4.5.3 *In vitro* glycemic response the encapsulates

In vitro digestion experiments were conducted to evaluate the amount of reducing sugar released during the whole digestion. Figure 4.2 demonstrates the amount of reducing sugar released over a 120-min digestion. There was a significant decrease in reducing sugar release for both SWB and FWB compared to their corresponding controls. Spray-drying and freeze-drying had no significant effects on the amount of reducing sugar released. Blackcurrant concentrate used in this study has a high content of available carbohydrate, including starch. The decrease in reducing sugar release can be attributed to inhibition of the amylase activity of anthocyanins in blackcurrant concentrate (Barrett, Farhadi, & Smith, 2018). As the digestion process went on, the protein particles were hydrolysed by pepsin, and the reducing sugar in the control group was gradually released. This could be an explanation for the increased the reducing sugar in control groups (SWC, FWC) (Moreno *et al.*, 2018).

4.5.4 Alpha-amylase inhibition activity of extracts from protein encapsulates

Alpha amylase is one of the major enzymes involved in the digestion of starchy food, releasing oligosaccharides that can be further degraded to glucose, which is rapidly absorbed by the body. Consequently, inhibition of α-amylase activity is considered to be an effective strategy for managing diabetes. Figure 4.2 presents the IC_{50} values of extracts towards α-amylase inhibitory activity. It is not surprising that acarbose exhibited the highest inhibitory activity, with its IC_{50} value 65.33 μg/mL since acarbose is a purified synthetic α-amylase inhibitor and is used as a positive control in this thesis (Channale *et al.*, 2016). The results showed that extracts from FWB exhibited the strongest inhibitory activity (IC_{50} = 73.46 μg/mL), followed by extracts from SWB (IC_{50} = 81.46 μg/mL), indicating that blackcurrant anthocyanins could be responsible for the α-amylase inhibitory activities of FWB extract and SWB extract.

Blackcurrant, due to its high polyphenol content, has been reported to have α-amylase inhibitory activity (Boath, Stewart, & McDougall, 2012). A previous study revealed that this inhibitory activity was mainly due to anthocyanidins (delphinidin 3-O-rutinoside, delphinidin 3-O-glucoside, cyanidin 3-O-glucoside, cyanidin 3-O-rutinoside) from blackcurrant (Hui *et al.*, 2020). In addition, the present study suggests that encapsulation by freeze-drying may be more effective than by spray-drying regarding the enzyme inhibitory activity due to the stronger inhibitory activity of freeze-dried sample extract. This observation is consistent with the high performance liquid chromatography results presented in Chapter 5 that freeze-dried sample extract has higher anthocyanins content. Freeze-drying process may avoid the negative impact of high temperature on thermally sensitive anthocyanins and entrap a higher anthocyanin content (Laokuldilok & Kanha, 2015). Most of the anthocyanin was well preserved inside the freeze-dried microcapsules. Therefore, here, we hold the opinion that freeze-drying might be a more efficient technique compared to spray-drying, for preservation of anthocyanidins that was responsible for the α-amylase inhibitory activity.

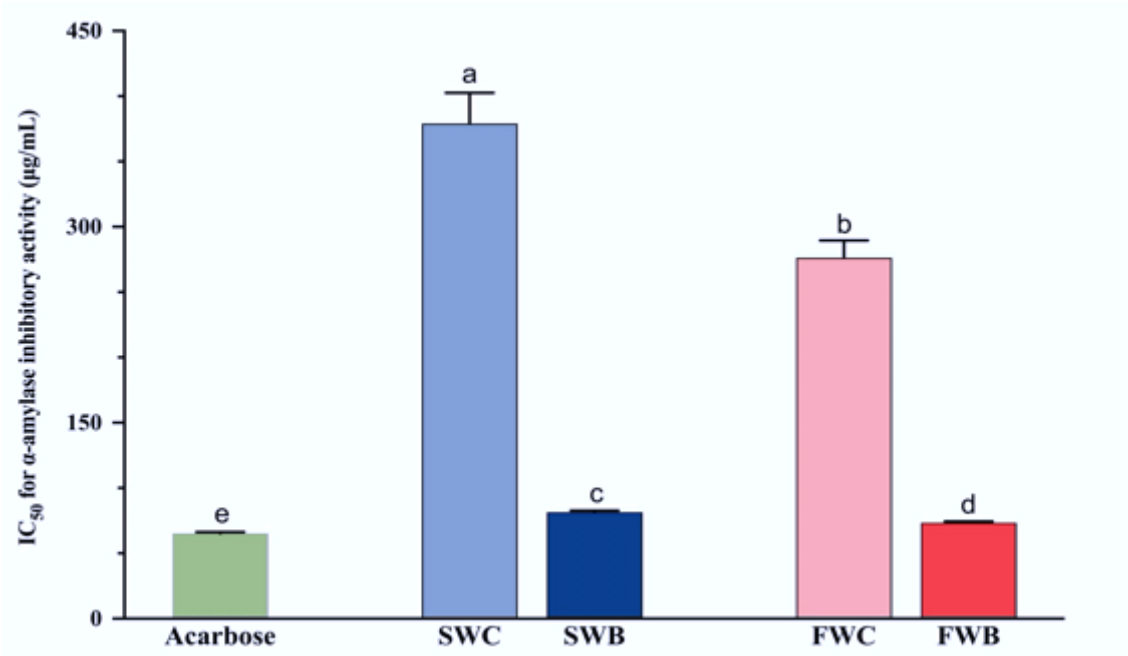

Figure 4.2 Alpha-amylase inhibitory activity of sample extracts.

Error bars indicate standard deviation (n = 3). Significant differences among samples are expressed by different lowercase letters. SWB: spray-dried whey protein isolate + blackcurrant concentrate; SWC: spray-dried whey protein isolate + imitation blackcurrant juice; FWB: freeze-dried whey protein isolate + blackcurrant concentrate; FWC: freeze-dried whey protein isolate+ imitation blackcurrant juice.

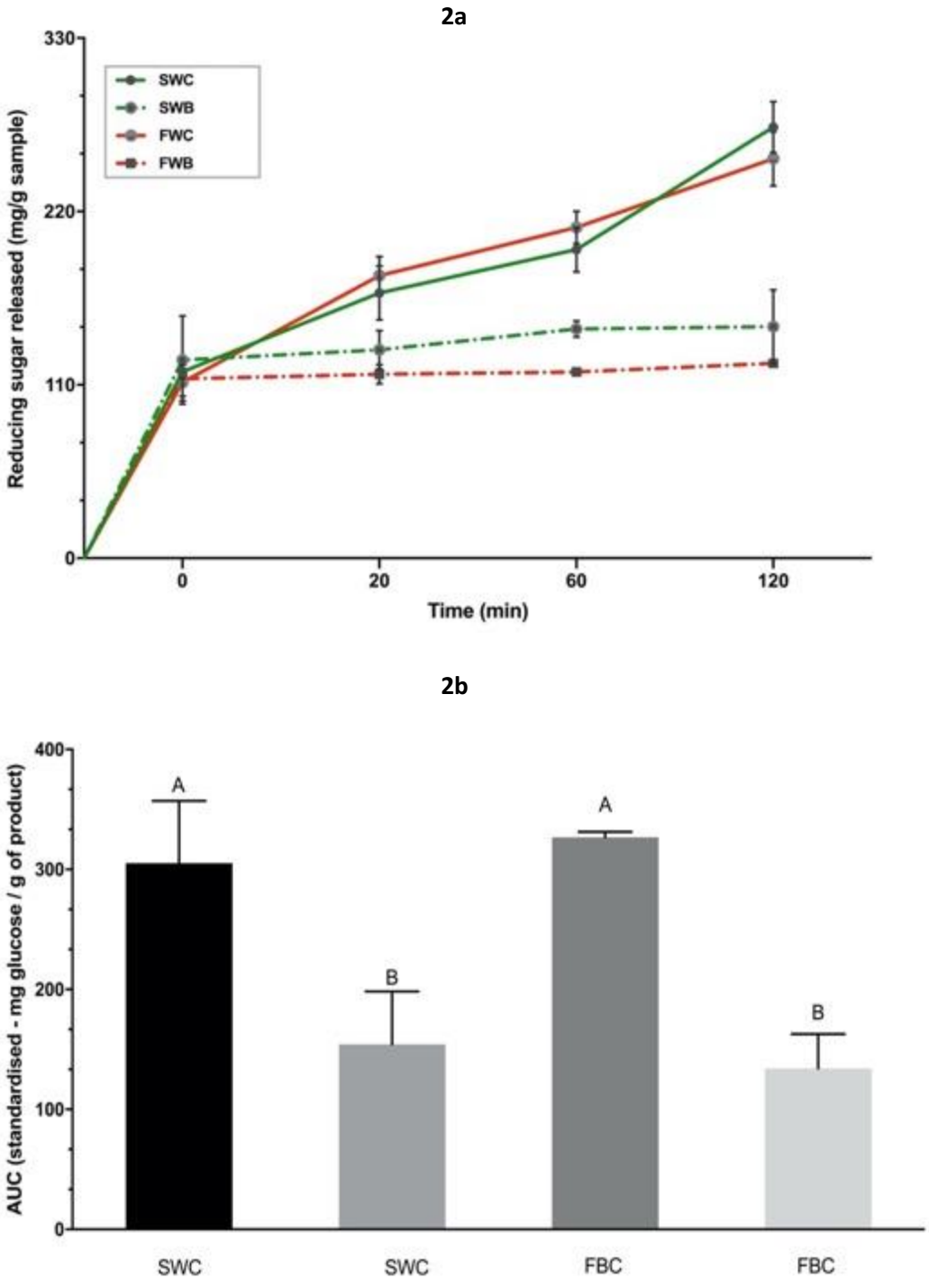

Figure 4.3 Reducing sugar released during *in vitro* digestion process

(2a) Reducing sugar content and (2b) the corresponding area under the curve. Different letters are significantly different from each other ($p < 0.05$). Error bars indicate standard deviation (n = 3).

4.5.5 Total phenolic content and antioxidant activity

Figure 4.4a shows that the total phenolic content value of SWB and FWB decreased after the intestinal digestion. This could be attributable to the degradation of phenolics (Pineda-Vadillo *et al.*, 2016), and potential interaction with amino acids, polypeptide chains or even protein molecules in the digest (Flores, Singh, Kerr, Pegg, & Kong, 2014). The changes in antioxidant activity of all samples during the *in vitro* digestion were evaluated by FRAP and DPPH assay (Figure 4.4b-4.4c). Overall, a positive correlation between total phenolic content and antioxidant activity was observed. These results could be attributed to the generation of antioxidative amino acids or protein peptides, and the release of sensitive bioactive compounds followed by degradation due to exposure to imitated digestive conditions. Spray-dried and freeze-dried products showed similar antioxidant activity distributions, and this was similar to other research (Fredes, Becerra, Parada, & Robert, 2018). However, spray-dried powder presented yielded higher TPC values, which may be attributed to higher encapsulation efficiency in spray-dried powder than freeze-dried, forming a better protective effect for the antioxidant components. These results were consistent with some previous studies. For instance, an *in vitro* study by Hoskin, Xiong, Esposito, and Lila (2019) concluded that spray-dried protein polyphenol particles had better biochemical activity. Rezende, Nogueira, and Narain (2018) reported that spray-dried treatment led to the retention of higher concentrations of bioactive compounds.

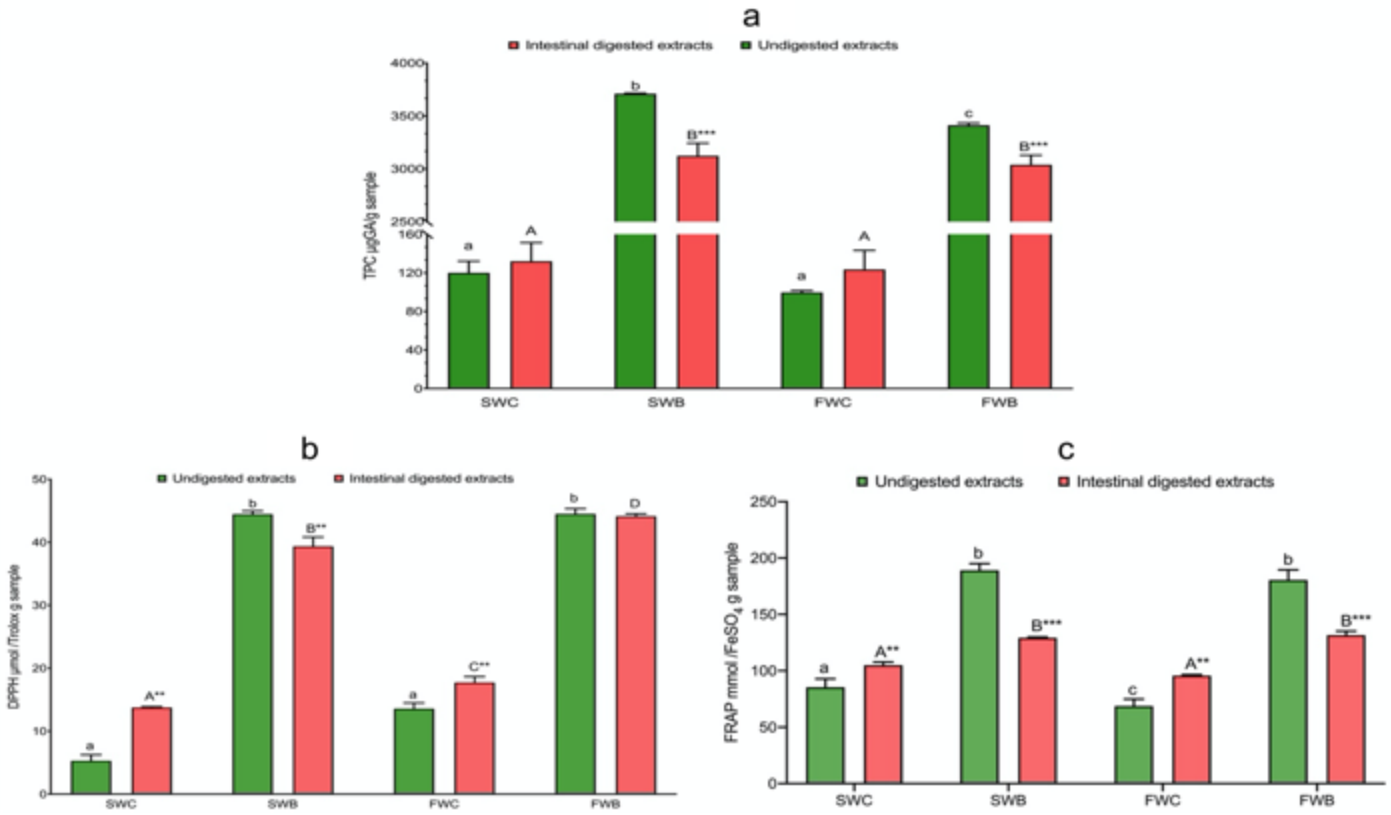

Figure 4.4 Total phenol content (TPC) of undigested extracts and intestinal digested extracts

*Significant difference among extracts is expressed by different lowercase letters, significant difference among digesta is expressed by different capital letters, and significant difference before and after digestion is indicated by the asterisk (*p < 0.05, **p < 0.01, ***p < 0.001). Error bars represent standard deviation (n = 3). SWB: spray-dried whey protein isolate + blackcurrant concentrate; SWC: spray-dried whey protein isolate + imitation blackcurrant juice; FWB: freeze-dried whey protein isolate + blackcurrant concentrate; FWC: freeze-dried whey protein isolate+ imitation blackcurrant juice*

4.5.6 Molecular docking study

Molecular docking is widely used to study the interactions between proteins and small molecules. This technique was used to investigate the interaction behaviours between the four main individual blackcurrant anthocyanins and α-amylase in this study to further validate the potential inhibition mechanisms of specific anthocyanins acting on α-amylase. The binding energy, number of hydrogen bonds, and the amino acid residues involved in hydrophobic interactions of the four anthocyanins with α-amylase were shown in Figure 4.5. Cyanidin 3-O-glucoside and cyanidin 3-O-rutinoside had a similar binding energy of -9.8 kcal/mol and -9.9 kcal/mol, respectively. Delphinidin 3-O-glucoside had the lowest binding energy of -10.3 kcal/mol, whereas delphinidin 3-O-rutinoside had the highest binding energy (-5.4 kcal/mol). The lower the binding energy, the easier it is for the ligand to bind with the protein (Sui *et al.*, 2018). Hence, cyanidin 3-O-glucoside, cyanidin 3-O-rutinoside, and delphinidin 3-O-glucoside were more likely to bind with α-amylase than delphinidin 3-O-rutinoside.

Cyanidin 3-O-rutinoside forms six hydrogen bonds with α-amylase, whereas cyanidin 3-O-glucoside forms one hydrogen bond with the enzyme. Delphinidin 3-O-glucoside forms three hydrogen bonds with α-amylase, while delphinidin 3-O-rutinoside forms five hydrogen bonds with the enzyme. Given that the hydrogen bonds formed between ligand and α-amylase differs for each anthocyanin and do not correlate with the predicated binding affinity, we believe that the formation of hydrogen bonds may be affected by chemical structures and glucosides types of each anthocyanin, a similar to the idea postulated by Nanashima, Horie, and Maeda (2018).

Amino acids residues involved in hydrophobic interactions are listed in Table 4.4, and their 2D structures are shown in Figure 4.5. It is worthy to note that delphinidin 3-O-glucoside, cyanidin 3-O-glucoside and cyanidin 3-O-rutinoside interact hydrophobically with residues, including Ser163(A), Leu162(A), Tyr62(A), His201(A), Trp59(A), and Trp58(A). Therefore, we believe that

delphinidin 3-O-glucoside, cyanidin 3-O-glucoside, and cyanidin 3-O-rutinoside bind within the same pocket, hindering the binding of α-amylase and starch molecules in the enzyme active sites, in agreement with the findings of Sui, Zhang, and Zhou (2016). The binding energies of delphinidin 3-O-glucoside, cyanidin 3-O-glucoside, and cyanidin 3-O-rutinoside also agree with this hypothesis given that their binding is more favourable than that of starch having a binding energy of -7.8 kcal /mol (Sin, Rahman, Rahmat, & Samad, 2010), when it binds in the α-amylase active site.

4.6 Conclusion

Blackcurrant juice concentrate functionalised whey protein isolate ingredients (SWB, FWB) have the potential to be utilised as novel protein ingredients, which are suitable to be applied in high carbohydrate products, to improve protein content, to reduce sugar addition, and to slow down the spiking of blood sugar. Meanwhile, blackcurrant functionalised whey protein isolate ingredients are natural colourants and antioxidants, which providing potential choices for innovative food products with better nutritional properties and sensory acceptance. Both spray-drying and freeze-drying could be potential options for the functionalisation of whey protein isolate with blackcurrant concentrate. In terms of practical application, spray-drying might be a better potion due to its higher encapsulation efficiency and industrial up scalability. Future research would focus on the inhibition activity on some other common enzymes (trypsin, pancreatin, α-amyloglucosidase), the alteration in functional properties of whey protein, such as gelling, emulsion, and foaming or the practical application of this functionalised protein ingredient in real food matrices, such as baking products.

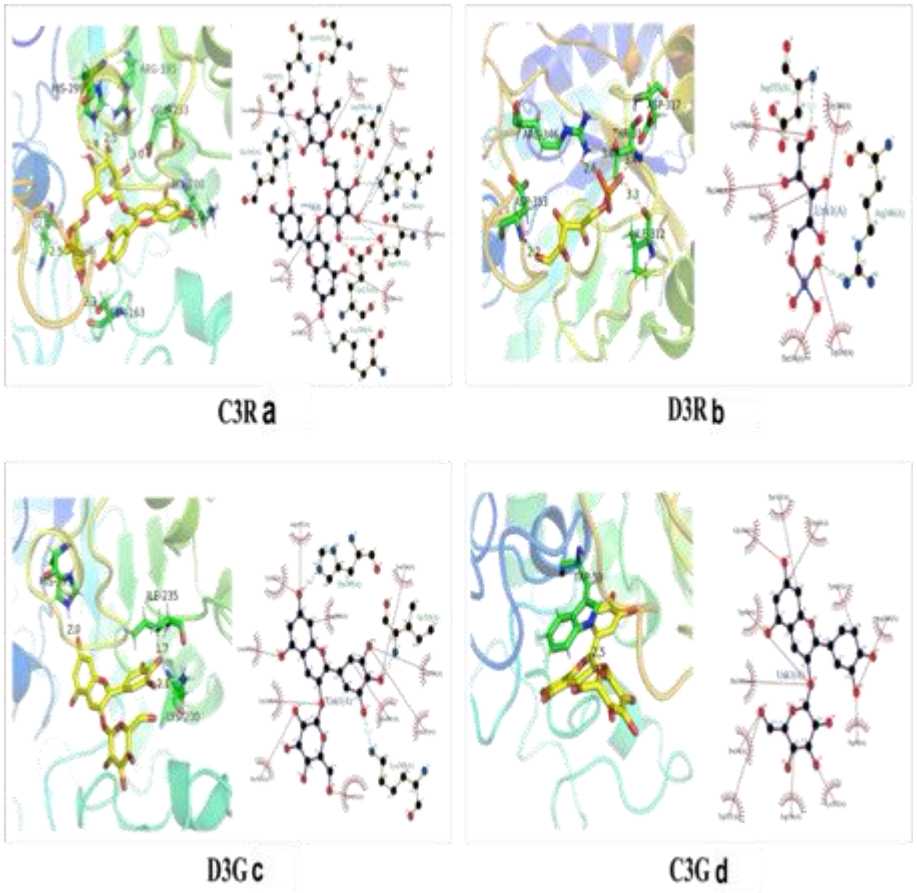

Figure 4.5 Four main blackcurrant anthocyanins bond in the α-amylase active sites.

D3R: delphinidin 3-O-rutinoside; D3G: delphinidin 3-O-glucoside; C3G: cyanidin 3-O-glucoside; C3R: cyanidin 3-O-rutinoside (C3R).

Table 4.4 Molecular docking results

Compound (CAS)	Affinity (kcal/mol)	Number of hydrogen bonds	Hydrophobic interaction with amino acid residues
D3G (6906-38-3)	-10.3	3	Tyr151(A), Ser163(A), Leu162(A), Leu165(A), Tyr62(A), Asp197(A), Asp300(A), Val234(A), Ala198(A), His201(A), Glu233(A)
D3R (15674-58-5)	-5.4	5	Thr314(A), Arg303(A), Phe348(A), Lys352(A), Gly304(A), Trp316(A)
C3G (7084-24-4)	-9.8	1	Asp356(A), Trp357(A), Pro54(A), His305(A), Trp59(A), Gly104(A), Ser163(A), Gln63(A), Tyr62(A), Asp300(A), Trp58(A), Lys352(A)
C3R (28338-59-2)	-9.9	6	Ile235(A), Leu162(A), Trp59(A), Trp58(A), Tyr62(A), Arg195(A), His201(A)

D3R: delphinidin 3-O-rutinoside; D3G: delphinidin 3-O-glucoside; C3G: cyanidin 3-O-glucoside; C3R: cyanidin 3-O-rutinoside

Chapter 5

Physical and functional characteristics, and anti-cancer properties of blackcurrant juice concentrate fortified whey protein isolate

(Published in *Food Chemistry*, DOI: 10.1016/j.foodchem.2021.129620)

5.1 Abstract

Novel protein ingredients were produced by encapsulating blackcurrant concentrate with whey protein through spray- and freeze-drying strategies. The effects of encapsulation strategies and the addition of blackcurrant concentrate on the physical and functional characteristics, and anti-cancer activity of the ingredients were evaluated. The interaction mechanisms of the blackcurrant anthocyanins with the whey protein components were investigated via *in silico* studies. High performance liquid chromatography results revealed that whey protein effectively delivered the blackcurrant anthocyanins. The physical and functional properties of the protein were altered by drying strategies and the addition of blackcurrant concentrate. Anti-cancer effects were linked to reactive oxygen species production and cell apoptosis. Molecular docking results showed that the hydrogen bonds were the main binding forces among blackcurrant anthocyanins and whey protein molecules, resulting in the formation of complexation. These findings are well relevant to the formulation of powdered products to be used as ingredients in food products and beverages.

5.2 Graphic abstract

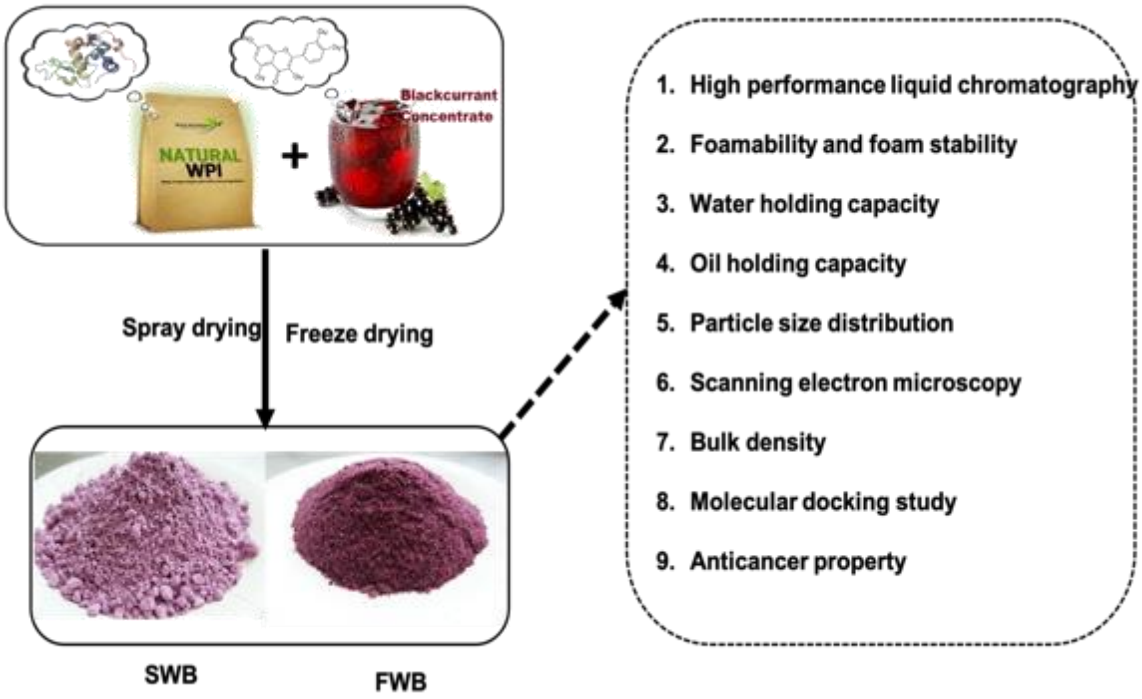

Figure 5.1 Sample preparation process and *in vitro* digestion conditions.

5.3 Introduction

Blackcurrant berry fruit has been shown to have many potential health benefits, such as antioxidant activity, protection of cardiovascular function, anti-microbial, anti-diabetes, anti-obesity, and anti-cancer effects (Azman, House, Charalampopoulos, & Chatzifragkou, 2020; Cortez & Gonzalez de Mejia, 2019; Gopalan *et al.*, 2012). Berry fruits have also been shown to be useful in the reduction of exercise-induced lactate accumulation, indicating the great potential of products containing blackcurrant for competitive athletes, not only participating in endurance sports, but also those requiring rapid and optimal muscle recovery (Willems, Myers, Gault, & Cook, 2015). These health benefits can be attributed to the abundant phytochemicals, especially anthocyanins in blackcurrant. Four main anthocyanins, responsible for the dark colour of the fruit, have been identified in blackcurrants, namely delphinidin 3-O-rutinoside, delphinidin 3-O-glucoside, cyanidin 3-O-glucoside, and cyanidin 3-O-rutinoside

(Bishayee *et al.*, 2010). These can be classified into two main forms without glucosides, namely delphinidin and cyanidin.

Whey protein is mainly composed of α-lactalbumin, β-lactoglobulin, bovine serum albumin, and lactoferrin, and has been widely accepted as an ingredient for protein supplements, especially for athletes (Lam *et al.*, 2019). One of the most common commercial forms of whey protein is whey protein isolate with its solution (5%, w/w) pH value of ~ 6.6. The isoelectric point of whey protein is 4.5. This is the point of maximal natural complexes formation between protein and anthocyanins molecules, owing to their natural binding affinities (Schneider *et al.*, 2016). Whey protein isolate has an excellent solubility through a wide range of pH conditions, which has positive effects on food processing and the human digestive system (Piccolomini, Kubow, & Lands, 2015). Except for direct consumption, whey protein has been widely used as wall material for sensitive food component encapsulation (Correia *et al.*, 2017; Darniadi, Ho, & Murray, 2018a). Protein molecules have natural binding affinities to anthocyanins through covalent and/or non-covalent interactions, leading to the formation of complexes, which, in return, affect the stability and bioavailability of bioactive molecules (Ozdal *et al.*, 2013). The integration of natural binding affinities to spray-drying or freeze-drying strategies makes it possible to increase the stability against microbial growth by converting liquid solutions into powders with low water content and activity (Roos, 2010). Thus, a combination of whey protein isolate with blackcurrant by means of a green synthesis manner has the potential to develop a novel edible protein ingredient (Ni *et al.*, 2020).

The most common commercial form of blackcurrant is blackcurrant concentrate with an extremely acidic pH value (pH ~ 2.9) (Cortez & Gonzalez de Mejia, 2019). The incorporation of blackcurrant concentrate into food matrices is a challenge faced by the food industry. Many aspects need to be considered, including stability and bioavailability. Blackcurrant antioxidants appear to be very stable and remain active after processing into juice, wine, and

jam (Cortez & Gonzalez de Mejia, 2019). It is also important to determine the bioaccessibility of these components once they are ingested and whether they have a protective effect when they get to a site of action (Jia, Li, Diao, & Kong, 2014). However, research examining the interactions of blackcurrant polyphenols with whey proteins and their further bioavailability is limited.

In Chapter 4, the physicochemical and nutritional properties of the protein encapsulate were evaluated. This chapter mainly focused on the physical properties (bulk density, particle size, and surface morphology), functional properties (foamability, foaming stability, water and oil holding capacity), as well as the potential anti-cancer activities of the novel protein ingredients towards the HepG2 cell line. In addition, a molecular docking study was employed to evaluate the interactions between whey components and anthocyanins.

5.4 Methods

5.4.1 Preparation of the protein ingredients

The ingredients (SWC, SWB, FWC, FWB) preparation was following the method described in 3.1.

5.4.2 High performance liquid chromatography analysis of the sample extract

High performance liquid chromatography analysis was conducted as the method described in 3.9.

5.4.3 Bulk density of encapsulates

The bulk density of the powdered ingredients was measured according to the method described in 3.17.

5.4.4 Particle sizes distribution

The particle sizes were measured by dynamic light scattering as described in 3.18.

5.4.5 Morphology properties

The surface morphology of the particles was observed by scanning electron microscopy following the protocol described in 3.16.

5.4.6 Foamability, Foam stability, and microscope structure of the foam

The foam formation capacity, foam stability, and microscope structure were following the methods described in 3.14.

5.4.7 Water holding capacity and oil holding capacity

Water holding capacity and oil holding capacity were measured as the methods described in 3.15.

5.4.8 HepG2 cell line study

HepG2 cell line study, including cell viability, cell apoptosis, reactive oxygen species, was carried out according to the description in 3.21 and 3.22.

5.4.9 Molecular docking study

Molecular docking studies was performed as described 3.23.

5.4.10 Statistical analysis

Statistical analysis was performed as described in 3.24.

5.5 Results and discussion

5.5.1 Validation of the delivery of the main anthocyanins in protein ingredients

Cyanidin and delphinidin, considered to be the most abundant anthocyanidins in blackcurrant, are known to interact with protein molecules and create numerous bio-functionalities (Bishayee *et al.*, 2010). High performance liquid chromatography was conducted on sample extracts to determine the anthocyanidins composition of the samples. As shown in Figure 5.2, cyanidin and delphinidin were detected in SWB at 265.20 ± 4.32 and 314.73 ± 3.80 μg/g,

respectively, while in FWB, the content of cyanidin and the content of delphinidin were 183.91 ± 0.05 and 217.00 ± 0.02 μg/g, respectively. These results revealed the stabilisation and effective delivery of blackcurrant anthocyanins by SWB and FWB. Spray-drying appeared to be a more appropriate encapsulation manner for blackcurrant concentrate compared with freeze-drying as SWB had higher blackcurrant anthocyanin content compared with FWB. The difference in the content of blackcurrant anthocyanins might be attributed to the unique particle properties of SWB and FWB, such as particle size, shape, and surface morphology, which could lead to different mechanisms of release during extraction in preparation for high performance liquid chromatography analyse.

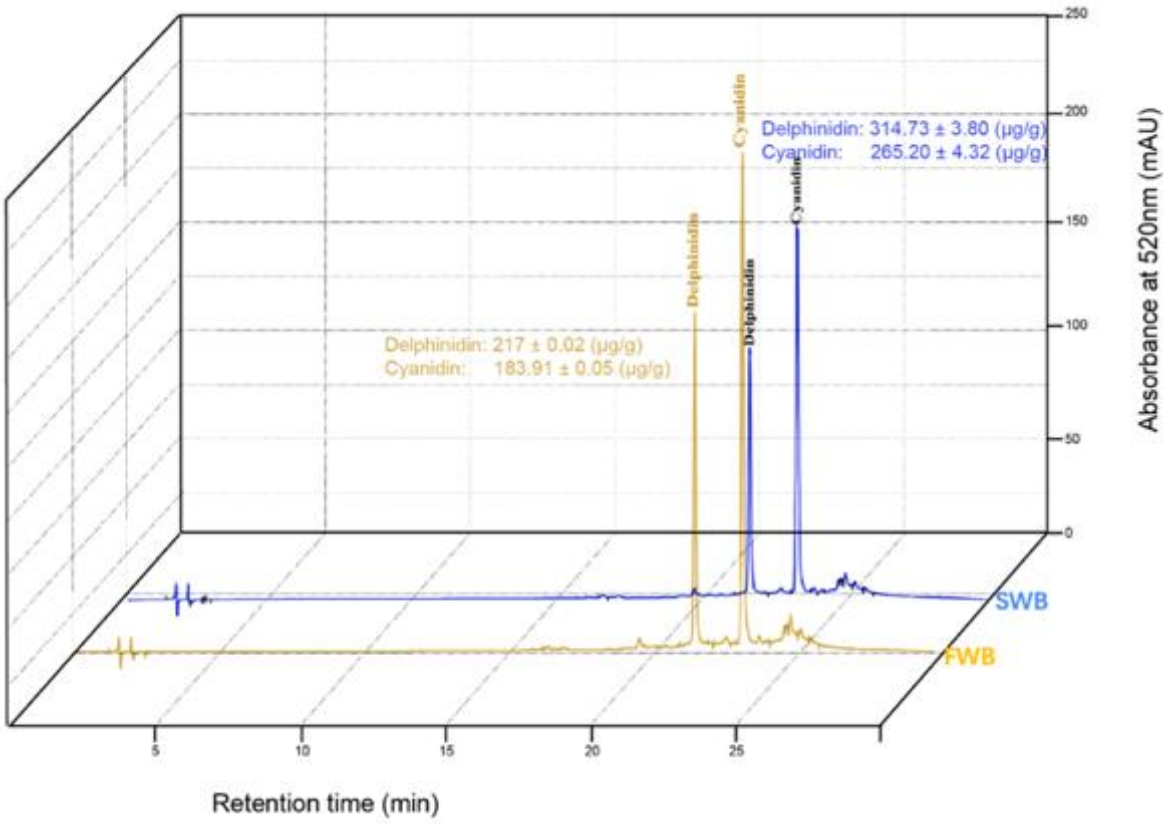

Figure 5.2 Concentration of cyanidin and delphinidin quantified by high performance liquid chromatography analysis.

SWB: spray-dried whey protein isolate + blackcurrant concentrate; FWB: freeze-dried whey protein isolate + blackcurrant concentrate

5.5.2 Alteration in physical properties

The development of different drying strategies resulted in unique physical properties of different particles, including bulk density, particle size distribution, morphology, and water holding capacity and oil holding capacity. Though SWB and FWB are not suitable for direct consumption, they can be used as food ingredients and subjected to further processing such as rehydration, extrusion, and mixing. Due to their complicated characteristics, such as bulk density, particle size distribution, morphology, and highly reactive physiochemical nature, it is essential to study the technical features of SWB and FWB ingredients to better understand their potential applications in food products (Schneider *et al.*, 2016).

Spray-dried ingredients are regular in terms of sizes and shapes. The particle sizes are mainly attributed to the physical conditions of spray-drying, including the atomiser, compressed air pressure and inlet temperature. On the other hands, freeze-dried ingredients are irregular in size and shape, which might be due to the feed solution concentration, the fragility of the formed flaky material, and the degree of grinding (Akbas *et al.*, 2017).

Bulk density is an important physical property, which can affect food transport, handling, and storage. The bulk density value depends on the size, shape, and surface characteristics of particles. Figure 5.3A shows the bulk density characteristics of the samples. Compared with whey protein isolate (0.334 ± 0.002 g/cm^3), both SWC and SWB demonstrated significantly lower ($p < 0.001$) bulk density values of 0.244 ± 0.003 g/cm^3 and 0.254 ± 0.001 g/cm^3, respectively. On the contrary, FWC and FWB showed significantly higher bulk density values ($p < 0.001$), of 0.383 ± 0.010 g/cm^3 and 0.406 ± 0.002 g/cm^3, respectively. There was a statistical difference ($p = 0.002$) between the bulk density values of FWC and FWB. This might be attributed to the increased fragility of the flaky structure due to the addition of blackcurrant concentrate.

Figure 5.3B illustrates the particle size characterisation of samples. The particle size of a product, and its distribution, are essential factors that can influence the bulk density, water holding capacity, oil holding capacity and their further incorporation of protein ingredients (Tonon, Grosso, & Hubinger, 2011). The irregular size and shape of freeze-dried ingredients may generate more external voids that can result in a higher bulk volume, which, in turn, leads to a lower bulk density (Rajam & Anandharamakrishnan, 2015). In contrast, spray-dried ingredients generally have a lower bulk density (Darniadi, Ho, & Murray, 2018b). This might be due to the hygroscopicity of the spray-dried powder, which could lead to the formation of a lump of powder (Figure 5.3C, indicated by the yellow circle). All the curves in Figure 5.3B exhibit monomodal size distributions. Table 5.1 shows the $D_{[4,3]}$ and the span values of the protein ingredients. On the one hand, the $D_{[4,3]}$ of SWC (15.94 ± 2.56 µm) and SWB (21.54 ± 2.17 µm) were significantly ($p < 0.001$) reduced by spray-drying, compared with original whey protein isolate (252.60 ± 6.43 µm). On the other hand, the $D_{[4,3]}$ of FWC (271.00 ± 6.78 µm) was significantly ($p < 0.001$) increased. The $D_{[4,3]}$ of FWB (219.40 ± 5.59 µm) was significantly ($p < 0.001$) decreased by freeze-drying compared to the original whey protein isolate. The lower $D_{[4,3]}$ of FWB compared to FWC might be due to an increased fragility of the flaky structure due to the addition of blackcurrant concentrate. The freeze-dried samples exhibited higher span values (2.59 - 3.04) when compared with the corresponding spray-dried samples ($p < 0.05$), indicating a wider range of particle size distribution and less homogeneity.

Figure 5.3C exhibits the morphology of the samples. Using a spray-drying strategy, the samples became a powder that was flour-like and moisture-absorbable, while the freeze-dried samples formed flaky powders. The morphology properties examined by scanning electron microscope further confirm the previous conclusions that spray-dried ingredients had larger particle sizes and irregular flaky shapes.

Figure 5.3D shows the water holding capacity and oil holding capacity of the ingredients. The water holding capacity of SWB and FWB ingredients were significantly ($p < 0.001$) higher compared to the corresponding control groups. The water holding capacity measurement is based on the amount of physical sedimentation under the effect of centrifugal forces, which is a direct reflection of protein denaturation and complex formation (Wasswa *et al.*, 2007). Both spray- and freeze-drying can lead to the denaturation of whey protein to some extent due to the extreme drying conditions, which can further result in the exposure of more ligand binding sites. The addition of blackcurrant concentrate improved the denaturation degree of the whey protein with the formation of larger complexes. Whey protein is insoluble in water, and the oil holding capacity is dependent on the voids between the particles. As shown in the images obtained from the scanning electron microscope (Figure 5.3C), freeze-dried samples formed more voids between particles, which is in agreement with the higher OHC of freeze-dried samples ($p < 0.001$).

Table 5.1 Particle size of the encapsulates

	$D_{[4,3]}$, (μm)	Span
WPI	252.60 ± 6.43[a]	1.99 ± 0.06[c]
SWC	15.94 ± 2.56[d]	1.57 ± 0.02[d]
SWB	21.54 ± 2.17[d]	1.59 ± 0.02[d]
FWC	271.00 ± 6.78[c]	2.59 ± 0.04[b]
FWB	219.40 ± 5.59[c]	3.04 ± 0.05[a]

WPI: whey protein isolate; SWB: spray-drying + whey protein isolate+ blackcurrant concentrate; SWC: spray-drying + whey protein isolate + imitation blackcurrant juice; FWB: freeze-drying + whey protein isolate + blackcurrant concentrate; FWC: freeze-drying + whey protein isolate+ imitation blackcurrant juice.

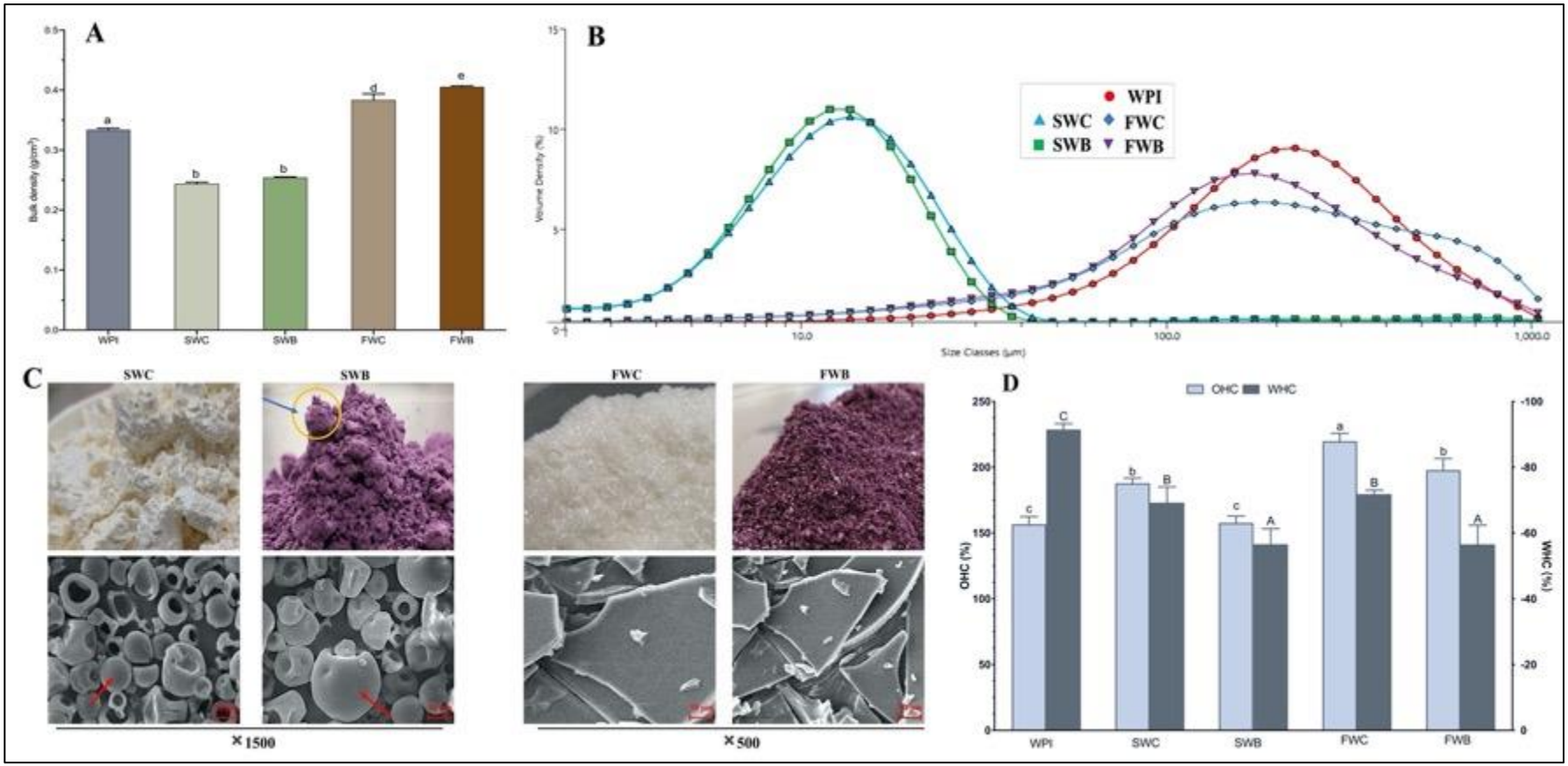

Figure 5.3 Physical characteristics of samples.

A: Bulk density (g/cm^3); B: Particle size distribution; C: Morphology of spray-dried and freeze-dried powders at x500 or x1,500 magnification; D: Water holding capacity (WHC) and oil holding capacity (OHC). Different letters are significantly different from each other ($p < 0.001$). Error bars represent standard deviation (n = 3).

5.5.3 Modifications in functional properties

Foam capacity and stability are important parameters for protein ingredients, which can reveal the potential of the protein ingredient to affect the quality and texture of products. The foaming capacity of a protein is based on the rapid diffusion of the partially unfolded protein at the air-water interface, which decreases the surface tension of the air bubbles (Wu, Clifford, & Howell, 2007). Once whey protein is mixed with blackcurrant concentrate, the blackcurrant anthocyanins in aqueous solution begin to alter the secondary and tertiary structures of the protein molecules via their natural binding affinity (Cheng, Liu, Prasanna, & Jing, 2017; Jia *et al.*, 2016). In addition, the physical conditions during the drying process, such as the atomisation and sudden exposure to high temperatures during spray-drying, and the freezing and sublimation during freeze-drying, have the potential to modify the secondary and tertiary structure of protein molecules (Zhan *et al.*, 2019). Therefore, when the dried protein ingredients are reconstituted, the newly exposed binding sites on the protein molecules further combine with the free water-soluble anthocyanin molecules. Thus, the original functionalities of whey protein, such as foaming capacity and stability, have already been altered.

Figure 5.4A depicts the microscopic profiles of the foam, providing the physical elucidation of the effects of blackcurrant anthocyanins on the foaming potential of whey protein isolate. The four formulated protein ingredients (SWC, SWB, FWC, and FWB) formed distinctly smaller (most less than 100 μm, as the red markers indicating on Figure 5.3A) air bubbles and more uniform bubble sizes compared to the whey protein isolate-based foam ($p < 0.05$). It was noticeable that FWB formed smaller bubbles than other groups ($p < 0.05$), which is consistent with the observation that FWB had the highest foamability. After standing for 30 min, the foam in FWB and SWB had more air bubbles than their corresponding control group ($p < 0.05$). Moreover, the FWB sample had comparatively more bubbles than the SWB sample ($p < 0.05$).

The morphological variations between the different foams assist in clarifying the differences in foaming capability and stability between samples.

Figure 5.4B illustrates the foaming capacity (0 min) and foam stability (30 min) of the protein ingredients. Spray- and freeze-drying techniques significantly increased the foaming ability of samples, in particular of FWB, with the highest foaming ability ($p < 0.05$). The most probable explanation for this result is that the drying conditions partially unfold the protein molecules, and then the addition of blackcurrant anthocyanins further unfolds the whey protein structures thoroughly, ultimately improving the foaming capacity of the protein particles (Cao, Xiong, Cao, & True, 2018).

In terms of foaming stability, SWB and FWB were significantly higher than their respective controls ($p < 0.05$), which can be attributed to the denaturation of natural protein molecules, and the further interaction between anthocyanins via covalent or non-covalent interaction (Sui *et al.*, 2018). A complex formation between denatured protein and anthocyanin molecules has been reported by Cao *et al.* (2018), which can lead to a thicker protein absorption on a film, benefiting the foam stability. The foam stability of FWB was significantly higher than SWB ($p < 0.05$). This might be attributed to more surface anthocyanins existing on the surface of freeze-dried particles. These free surfaces anthocyanins are involved in foam formation and are beneficial for reducing the surface tension of the bubbles.

5.5.4 Molecular docking results analysis

Four major protein types in the whey protein isolate, namely α-lactalbumin, β-lactoglobulin, bovine serum albumin, and lactoferrin, were selected as model proteins to dock with the four major blackcurrant anthocyanins, namely delphinidin 3-O-rutinoside, delphinidin 3-O-glucoside, cyanidin 3-O-glucoside, and cyanidin 3-O-rutinoside, separately. The docking results, including interaction energy, H-bonds, and possible binding sites, are presented in Table 5.2. As shown in Figure 5.5, Figure 5.6, Figure 5.7, and Figure 5.8, each protein provided

different binding sites for each anthocyanin molecule, apart from lactoferrin, which provided the same binding site for cyanidin 3-O-glucoside and delphinidin 3-O-glucoside. That is to say, the binding conformation appeared to be mainly decided by the properties of both macromolecules and corresponding ligands. The results also revealed that the predicted interaction energy of binding was different for each ligand and molecule, even for the interaction of lactoferrin with cyanidin 3-O-glucoside and delphinidin 3-O-glucoside. These results also indicated the involvement of other probable intermolecular interactions. Comparing the interaction energy values of each protein to the four anthocyanins molecules, lactoferrin exhibited the strongest interaction with the four anthocyanins, followed by bovine serum albumin > α-lactalbumin > β-lactoglobulin. The results are consistent with previous studies, which have reported that lactoferrin and bovine serum albumin might be the "target" of therapeutically active phenolics, and the two proteins have a strong correlation with phenolic metabolism (Cheng *et al.*, 2018; Rezende *et al.*, 2018). The 3D docking models reveal that many hydrophobic amino acid residues in the protein molecules provides the critical stabilisation of these protein-polyphenol complexes through hydrophobic interactions.

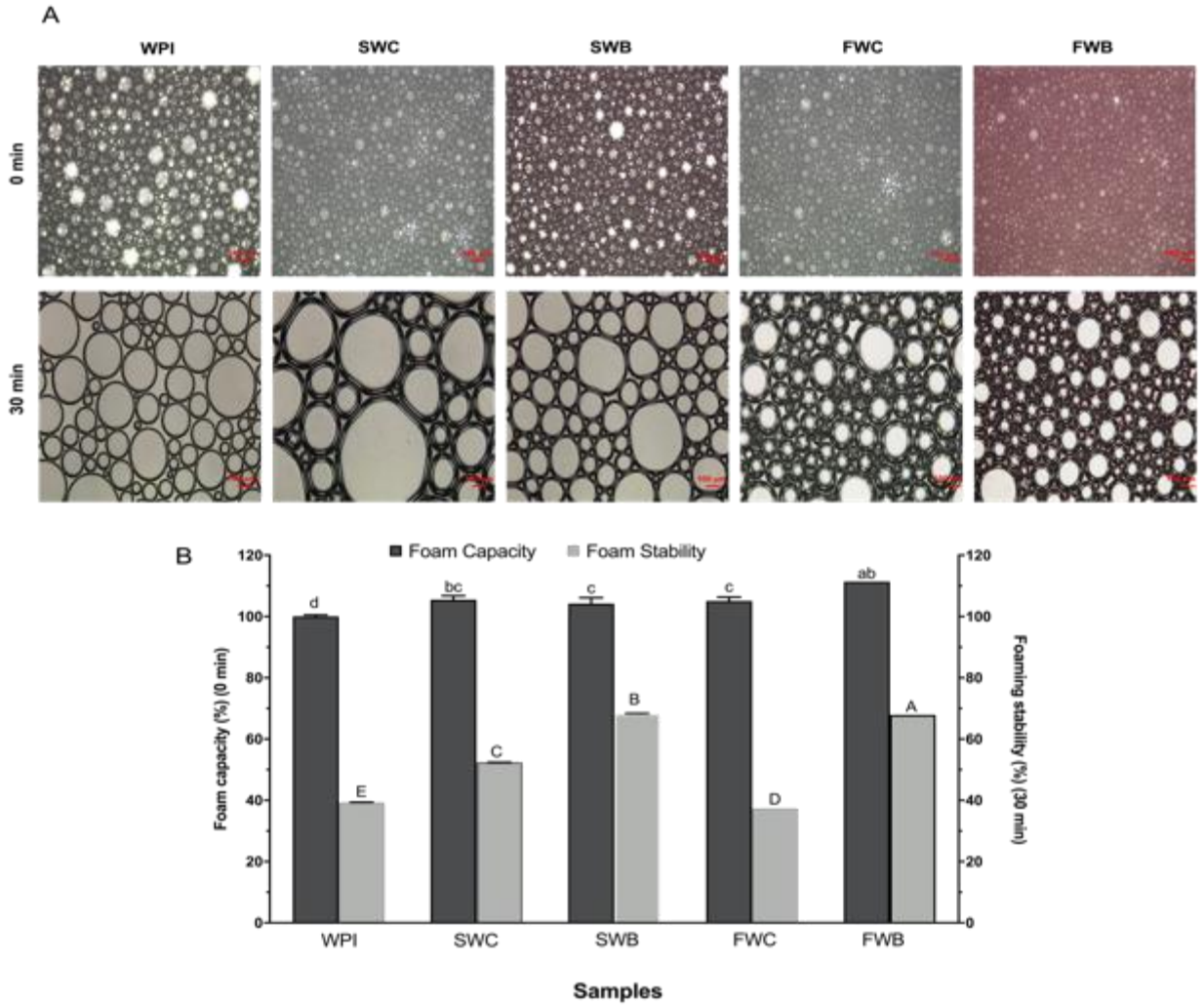

Figure 5.4 Microscope structures of the foam and foamability.

(A) Microscope structures of the foam (scale: 100 µm), (B) Foamability and foam stability. Significant differences among foam capacity are expressed by lowercase letters, significant differences among foam stability are expressed by capital letters. Error bars represent standard deviation (n = 3)

5.5.5 Anti-cancer effects on HepG2 via reactive oxygen species

SWB and FWB exhibited anti-cancer activity towards HepG2 cells. Figure 5.10A demonstrates that the HepG2 cell viability was suppressed by the SWB and FWB extracts in a concentration-dependent manner. The extrapolated IC_{50} values of SWB and FWB were 187.85 μg/mL and 211.08 μg/mL, respectively. This anti-cancer activity could be due to the blackcurrant anthocyanins from the extracts. This is in agreement with the conclusion in Chapter 6, which reported the inhibition of HepG2 cell proliferation by blackcurrant powder-enriched cookies due to the polyphenols from blackcurrant. Hossain *et al.* (2018) also stated that blackcurrant powder fortified cookie products have the potential to inhibit cancer cell growth.

SWB exhibited significantly stronger inhibitory activity than FWB ($p < 0.05$). Based on our preliminary tests, we decided on an inhibitory concentration of 200 μg/mL, for ROS generation and cell apoptosis analysis. Figure 5.10B shows that SWB and FWB induced ROS production, as shown by the shift of their respective peaks' positions to the right, compared with the control peak. Meanwhile, SWB induced a relatively higher generation of reactive oxygen species than FWB ($p < 0.05$). Figure 5.10C displays the HepG2 cell apoptosis results after exposure to 200 μg/mL of FWB/SWB for 24 h. Treatment with either SWB or FWB strongly induced HepG2 cell apoptosis ($p < 0.05$) compared with the control group (Figure 5.9). However, there was no significant difference between their late apoptosis effects ($p < 0.05$). In previous studies, blackcurrant extract was reported to have protective effects on the reactive oxygen species scavenging activity administrated at a certain concentration on the normal cells (Jia *et al.*, 2014). By contrast, our results showed that when HepG2 cell was treated with the concentration of IC_{50} of extracts, it could induce the generation of the intracellular reactive oxygen species, and further lead to the HepG2 cell apoptosis. This might be related to the difference in the biological characteristics between normal and cancer cells (Neves *et al.*, 2019).

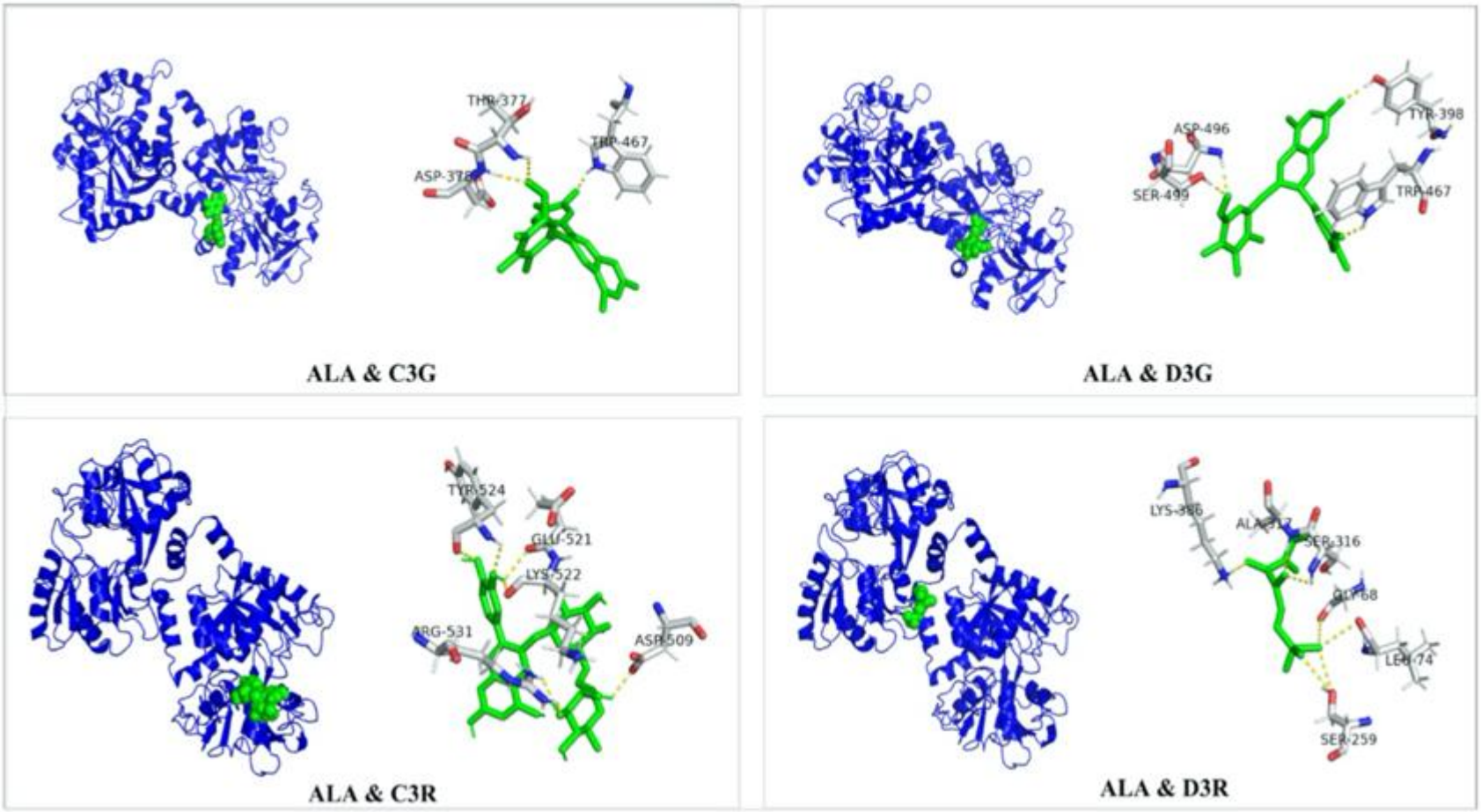

Figure 5.5 Molecular docking - ALA docked with the four main blackcurrant anthocyanins

The 3D structures represent conformational structures (left) and the hydrogen bonding (right) between protein molecules and the blackcurrant anthocyanins molecules. ALA: α-lactalbumin; BLG: β-lactoglobulin; BSA: bovine serum albumin; Lf: Lactoferrin; C3G: cyanidin 3-O-glucoside; C3R: cyanidin 3-O-rutinoside; D3G: delphinidin 3-O-glucoside; D3R: delphinidin 3-O-rutinoside.

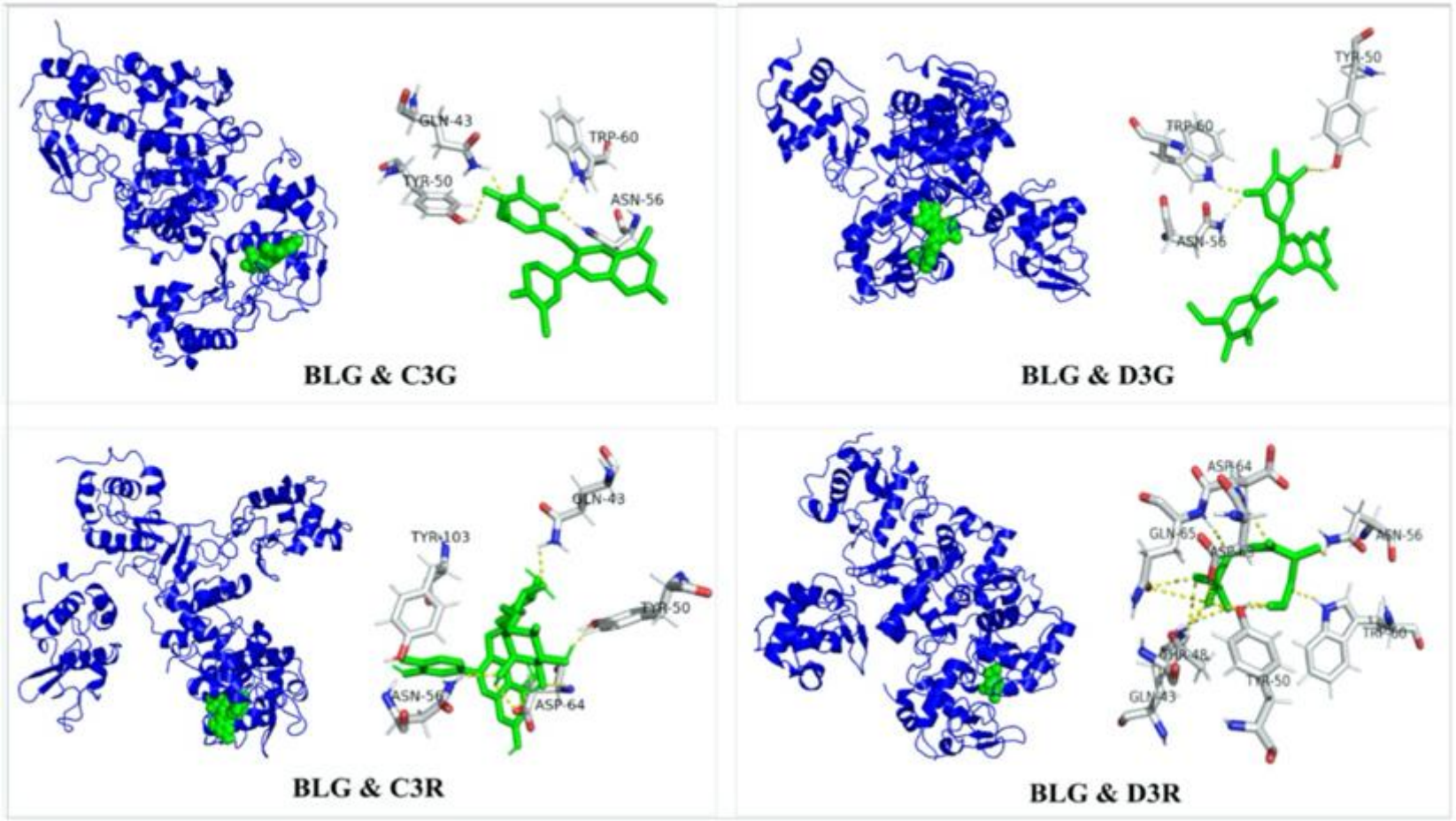

Figure 5.6 Molecular docking - BLG docked with the four main blackcurrant anthocyanins.

The 3D structures represent conformational structures (left) and the hydrogen bonding (right) between protein molecules and the blackcurrant anthocyanins molecules. ALA: α-lactalbumin; BLG: β-lactoglobulin; BSA: bovine serum albumin; Lf: Lactoferrin; C3G: cyanidin 3-O-glucoside; C3R: cyanidin 3-O-rutinoside; D3G: delphinidin 3-O-glucoside; D3R: delphinidin 3-O-rutinoside.

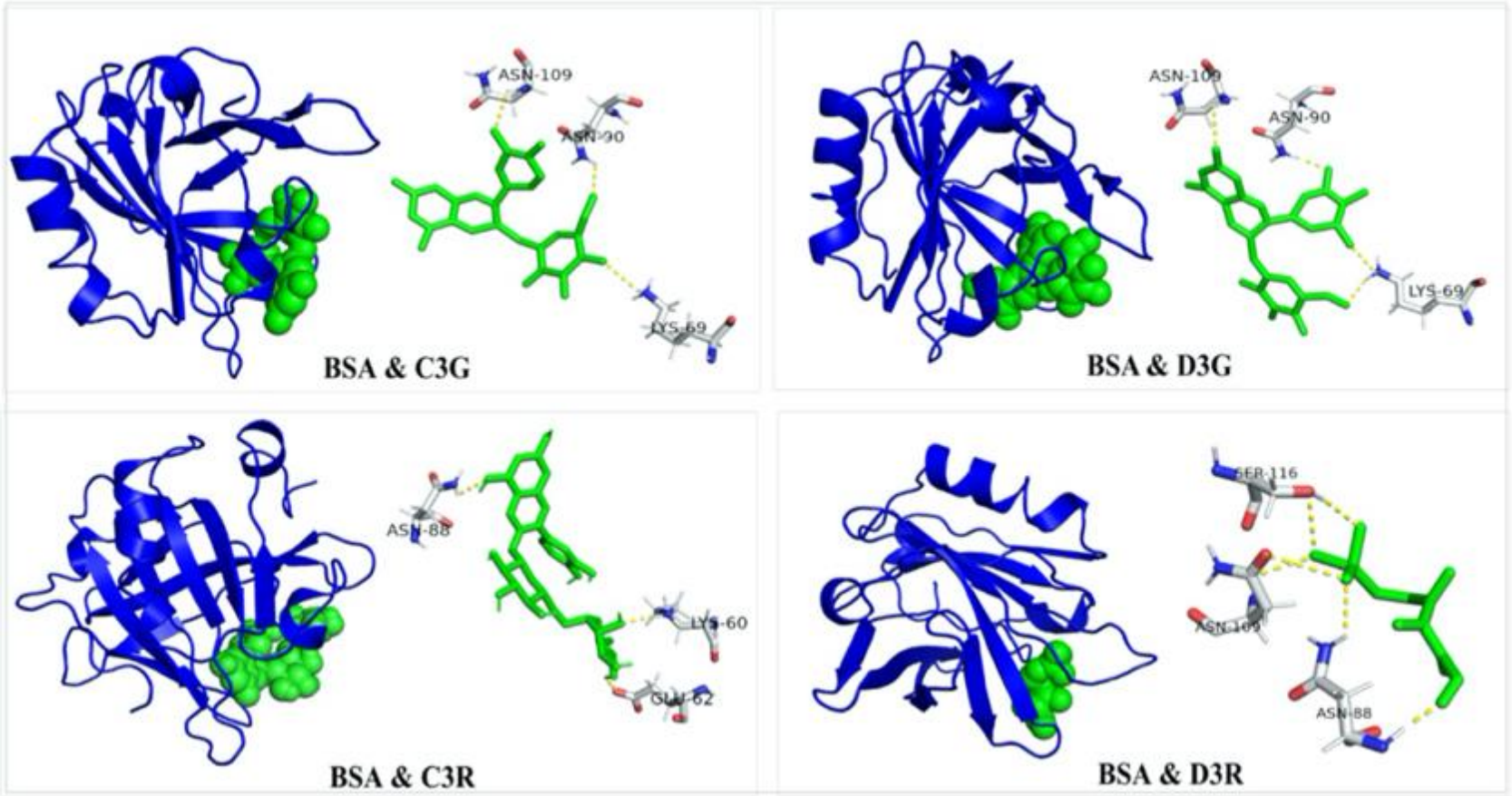

Figure 5.7 Molecular docking - ALA docked with the four main blackcurrant anthocyanins

The 3D structures represent conformational structures (left) and the hydrogen bonding (right) between protein molecules and the blackcurrant anthocyanins molecules. ALA: α-lactalbumin; BLG: β-lactoglobulin; BSA: bovine serum albumin; Lf: Lactoferrin; C3G: cyanidin 3-O-glucoside; C3R: cyanidin 3-O-rutinoside; D3G: delphinidin 3-O-glucoside; D3R: delphinidin 3-O-rutinoside.

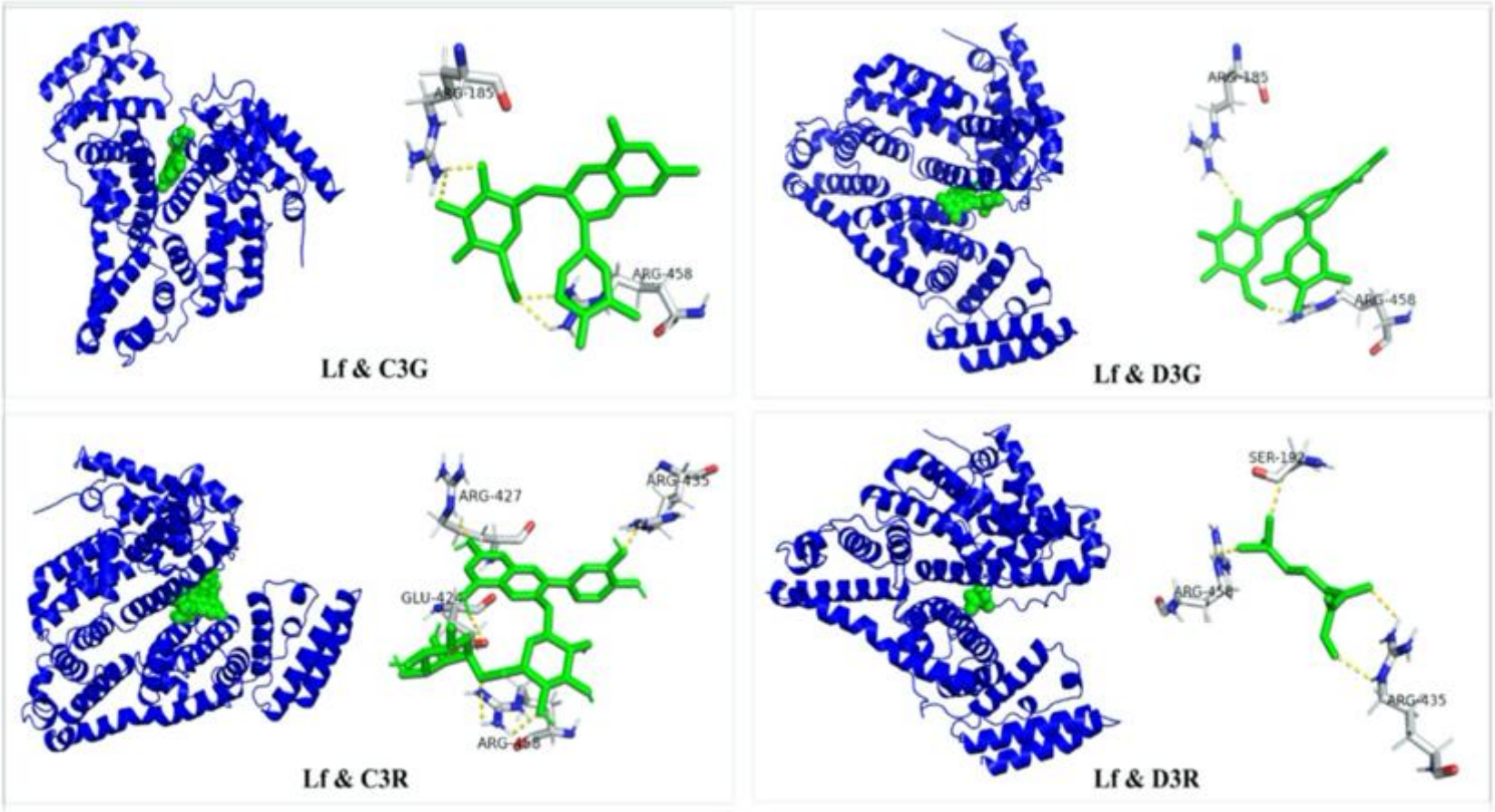

Figure 5.8 Molecular docking - LF docked with the four main blackcurrant anthocyanins

The 3D structures represent conformational structures (left) and the hydrogen bonding (right) between protein molecules and the blackcurrant anthocyanins molecules. ALA: α-lactalbumin; BLG: β-lactoglobulin; BSA: bovine serum albumin; Lf: Lactoferrin; C3G: cyanidin 3-O-glucoside; C3R: cyanidin 3-O-rutinoside; D3G: delphinidin 3-O-glucoside; D3R: delphinidin 3-O-rutinoside.

Table 5.2 Amino acid residues, number of H-bonds, binding affinity involved in the interaction of whey protein individual components with blackcurrant anthocyanins

Protein Name	PDB ID	Anthocyanins CAS	Affinity (kcal/mol)	Number H-bonds	Involved Residues
ALA	1F6S	7084-24-4(C3G)	-9.7	6	Asp-378, Thr-377, Trp-467
		28338-59-2(C3R)	-9.3	3	Lys-386, Ala-317, Ser-316, Gly-68, Leu-74, Ser-259
		6906-38-3(D3G)	-9.5	7	Ser-499, Asp-496, Tyr-398, Trp-467
		15674-58-5(D3R)	-5.1	4	Arg-531, Tyr-524, Glu-521, Lys-522, Asp-509
BLG	3NPO	7084-24-4(C3G)	-10.2	6	Tyr-50, Gln-43, Trp-60, Asn-56
		28338-59-2(C3R)	-9.2	4	Gln-43, Thr-48, Gln-65, Asp-63, Asp-64, Asn-56, Trp-60, Tyr-50
		6906-38-3(D3G)	-10.5	13	Asn-56, Trp-60, Tyr-50
		15674-58-5(D3R)	-5.7	3	Asn-56, Tyr-103, Gln-43, Tyr-50, Asp-64
BSA	4F5S	7084-24-4(C3G)	-8.8	3	Asn-109, Asn-90, Lys-69
		28338-59-2(C3R)	-8.1	3	Asn-109, Ser-116, Asn-88
		6906-38-3(D3G)	-9	6	Asn-10, Asn-90, Lys-69
		15674-58-5(D3R)	-4.6	4	Asn-88, Lys-60, Glu-62
Lf	1BLF	7084-24-4(C3G)	-11.3	5	Arg-185, Arg-458
		28338-59-2(C3R)	-10	4	Arg-458, Ser-192, Arg-435
		6906-38-3(D3G)	-12.7	4	Arg-185, Arg-458
		15674-58-5(D3R)	-5.8	2	Glu-424, Arg-427, Arg-435, Arg-458

ALA: α-lactalbumin; BLG: β-lactoglobulin; BSA: bovine serum albumin; Lf: Lactoferrin; C3G: cyanidin 3-O-glucoside; C3R: cyanidin 3-O-rutinoside; D3G: delphinidin 3-O-glucoside; D3R: delphinidin 3-O-rutinoside.

5.6 Conclusion

Both spray-drying and freeze-drying have effectively encapsulated blackcurrant concentrate anthocyanins using whey protein isolate as a carrier. Different encapsulation strategies resulted in encapsulates with different physical and functional characteristics. Definition of their specific physical and functional characteristics is essential for further application of these ingredient in an appropriate food matrix. Anti-cancer activity of the novel protein ingredients (SWB and FWB) was confirmed by the inducement of ROS generation and further inducement of cancer cell apoptosis. Molecular docking results indicated the interaction mechanism at a molecular level. Future research maybe focused on the incorporation of these novel functional protein ingredients into a real food product.

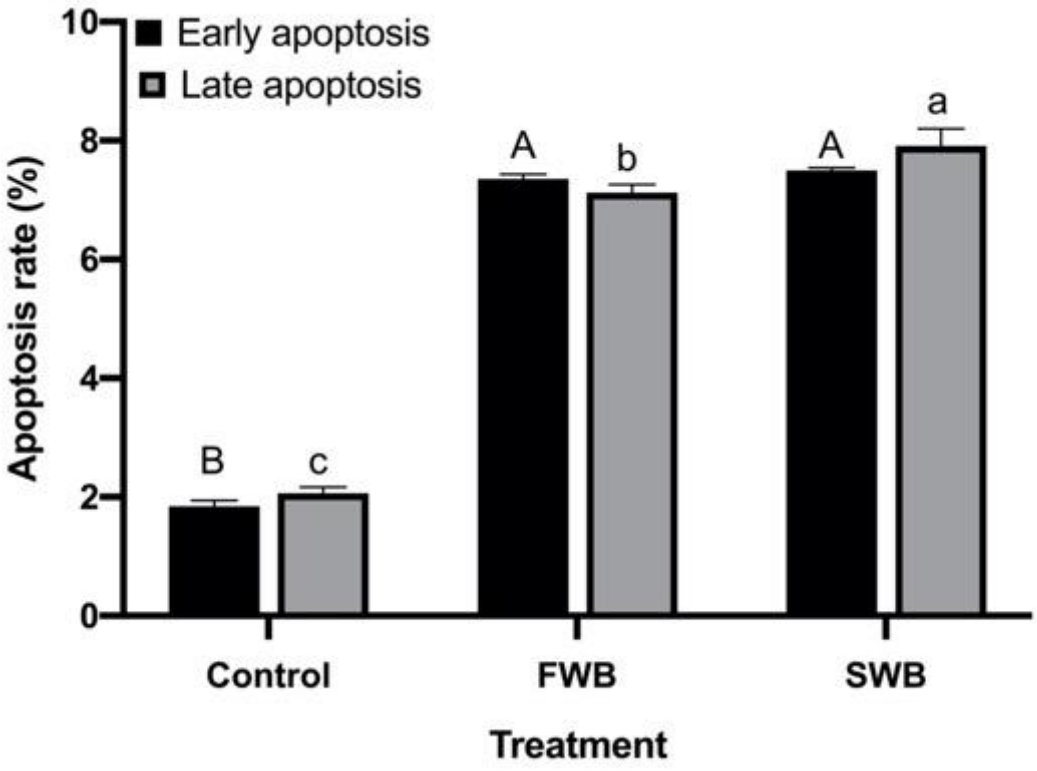

Figure 5.9 Quantitative analysis of HepG2 cell apoptosis.

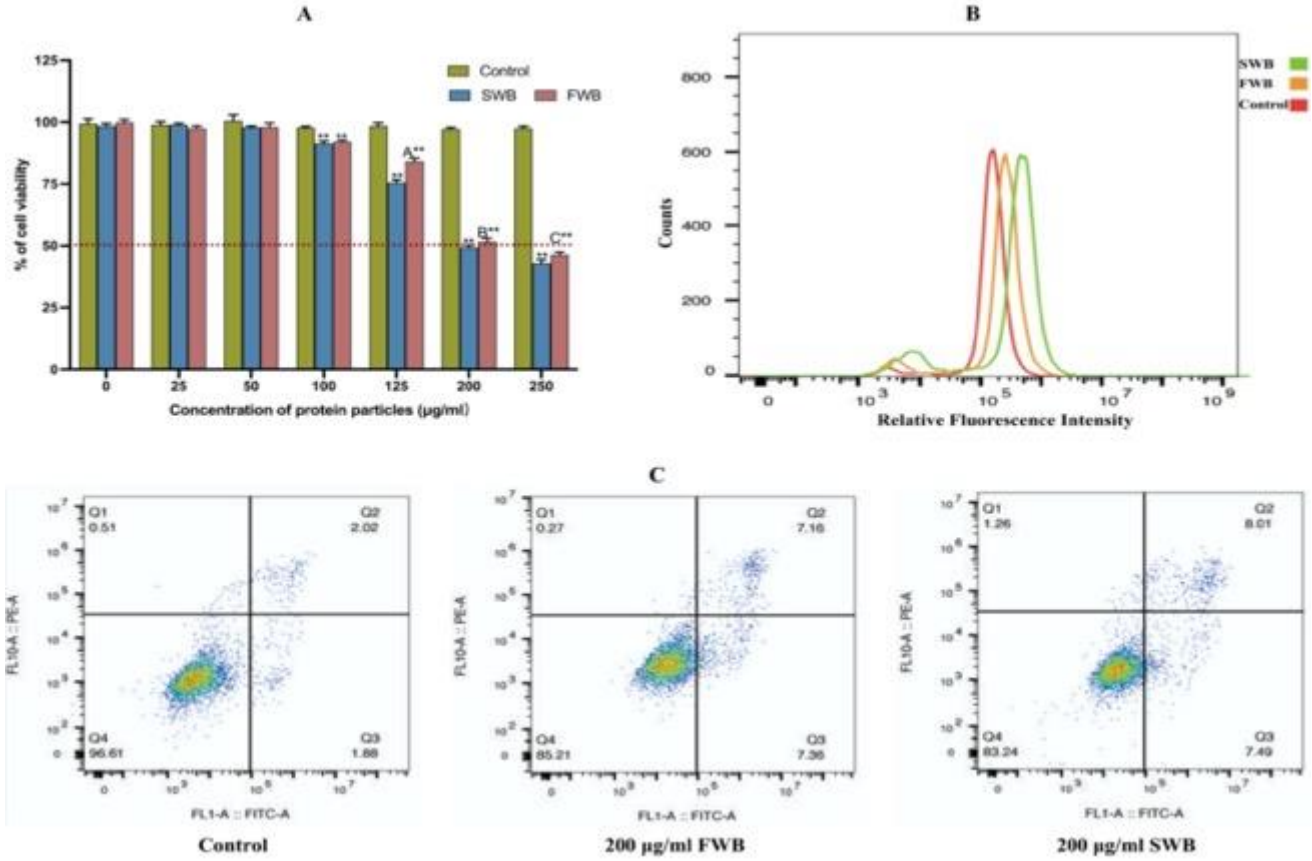

Figure 5.10 Anti-cancer attributes of SWB and FWB.

A: HepG2 cell viability (%) after administration of SWB and FWB. Significant differences among different concentrations are expressed by capital letters, Significant differences between SWB and FWB are indicated by the asterisk ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Error bars represent standard deviation (n = 3). B: Reactive oxygen species (ROS) production by flow cytometry analysis. C: Induction of HepG2 cell apoptosis by flow cytometry analysis.

Chapter 6

Incorporation functional protein ingredients into cookies to develop functional snacks

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6.1 Abstract

Milk whey can interact with polyphenols leading to the formation of complexes. In Chapter 4 whey protein isolate was formulated with blackcurrant concentrate by spray-drying (SWB) and freeze-drying (FWB), separately. Both SWB and FWB exhibited the potential to be used as functional ingredients. Therefore, in this chapter, SWB and FWB were incorporated in cookie dough, replacing 0%, 5%, 10% and 15% of flour to uncover the effects on physicochemical and nutritional properties of cookie products. The colour and texture parameters of cookies were influenced by the amount of protein ingredients added. Combination of the protein ingredients with cookies provided a higher protein and lower carbohydrates diet. The total phenolic contents in SWB-enriched cookies were lower than the corresponding FWB-enriched cookies, but higher than the control cookies. FWB-enriched cookies showed stronger ($P < 0.05$) radical scavenging capacity and reducing power than the corresponding SWB-enriched cookies before the digestion. After the intestinal digestion, FWB-enriched cookies decreased and showed lower reducing power, but higher ($P < 0.05$) radical scavenging capacity than the corresponding SWB-enriched cookies. FWB-enriched cookies exhibited stronger ($P < 0.05$) hypoglycaemic properties than SWB-enriched cookies. Blackcurrant concentrate fortified whey protein ingredients have positively influenced the functional characteristics of fortified cookies.

6.2 Graphic abstract

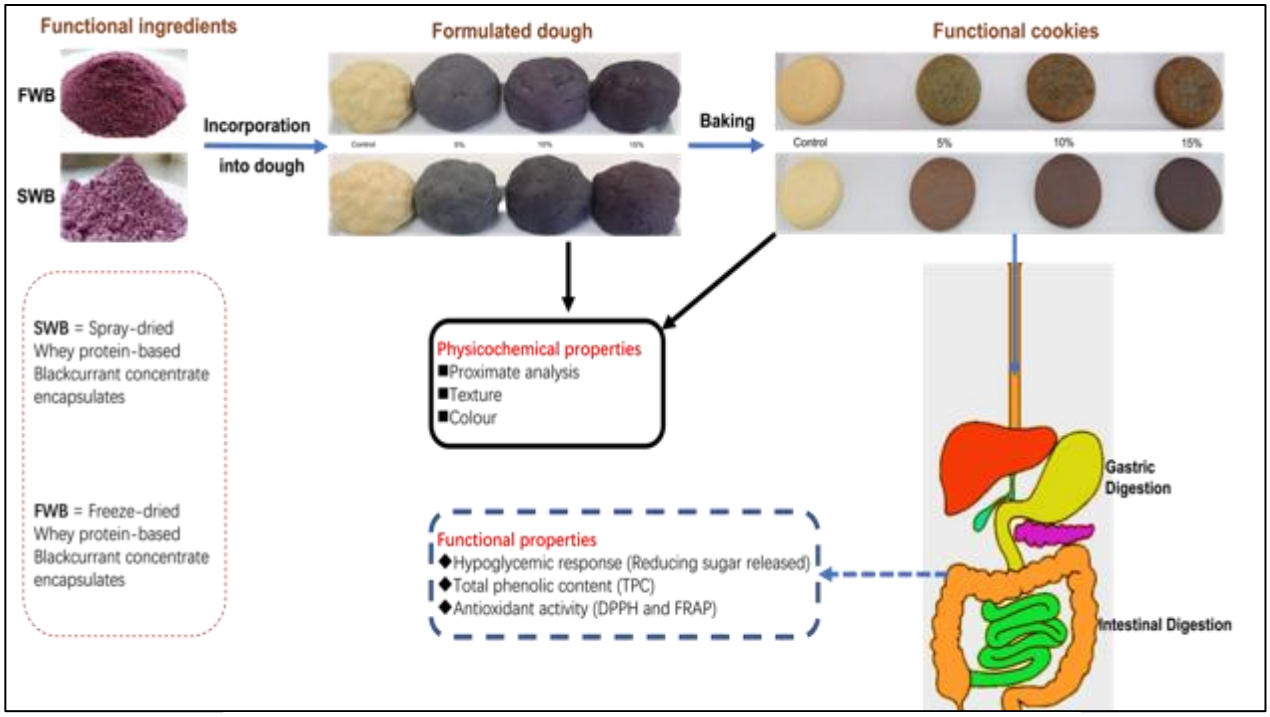

Figure 6.1 Cookie production and *in vitro* digestion.

6.3 Introduction

Soaring chronic diseases such as heart disease, cancer, and diabetes have been defined as a global pandemic and attracted increasing attention by the general population (Lou *et al.*, 2020; Yeganeh, 2019). Epidemiological studies have revealed that diet is a leading modifiable determinant of major chronic diseases. Increasing intake of dietary polyphenols appropriately is beneficial to combat all kinds of common chronic diseases (Cena & Calder, 2020). The prevalence of diabetes in the population has raised concerns about the intake of dietary carbohydrate and the modulation of postprandial blood glucose levels (Seckold, Fisher, de Bock, King, & Smart, 2019). Currently, people are keen on consuming a functional product with high protein content, low carbohydrate, and extra health benefits, but without compromise in cost and palatability (Katz & Meller, 2014; Li, Ho, Hayes, & Ferruzzi, 2019).

Numerous studies have investigated the functionalisation of whey protein isolate by combining whey protein isolate with fruits and vegetable concentrates or extracts rich in phenolic compounds (Ni *et al.*, 2020; Souza *et al.*, 2018; Tavares, Barros, Vaghetti, & Noreña, 2019; Tsali & Goula, 2018). Apart from protecting phenolic compounds from heat, light, and oxygen, whey protein isolates have been shown to give health benefits of concentrates or extracts (Raikos *et al.*, 2019; Tsali & Goula, 2018). The obtained products were further defined with specific physicochemical and nutritional properties and proposed as a potential functional ingredient as a colourant, protein enhancer, carbohydrate replacement, antioxidants (Hu, Li, Zhang, Kou, & Zhou, 2018). As described in chapter4, it has developed functional protein ingredients in a food-compatible manner by combining whey protein isolate with blackcurrant concentrate to form complexes and/or conjugates. Spray-drying and freeze-drying strategies were used to transform the liquid solutions into powders for easier handling, resulting in two protein powders (SWB and FWB, respectively) with their unique properties, including colour, particle morphology, protein content, oil holding capacity, water holding ability, potent antioxidant activity, and glycaemic response.

Previously whey protein isolate combined with blackcurrant concentrate has been shown to enhance the stability, antioxidant activity and *in vitro* release of polyphenols by delivering the bioactive compounds to lower parts of the gastrointestinal tract, increasing their bioavailability and bioaccessibility (He, Yuan, Zeng, & Chen, 2015; Huang *et al.*, 2019). However, very few studies have dealt with their incorporation in real food systems. When considering the further application of these functional ingredients, the fact that the functional components, such as anthocyanins, encapsulated within these ingredients are still water-soluble in an aqueous solution (Mäkilä *et al.*, 2016). Thus, the choice of an appropriate food matrix is of vital importance. A solid food matrix, such as dough for cookies or bread, provides an option.

For this reason, a snack food (a cookie was used as a simple model food) that provides macronutrients (protein and carbohydrate) with extra health benefits of blackcurrant has been developed, improving the quality of diet. This chapter was designed to investigate the possibility of developing a functional cookie, supplemented with encapsulated blackcurrant concentrate polyphenols. Different replacement levels of encapsulated blackcurrant concentrate polyphenols (0%, 5%, 10%, 15%) for wheat flour were investigated to understand their effects on dough and cookie properties. The effects on physicochemical properties (colour, texture, proximate) and nutritional characteristics (total phenolic content, antioxidant activity, and *in vitro* glycaemic response) of cookies were examined.

6.4 Methods

6.4.1 Cookie production

Cookie samples were prepared according to the protocol in 3.2.

6.4.2 Textural properties of dough and cookies

Texture measurement was carried out by following the methods described in 3.19.

6.4.3 Colour profiles of dough and cookies

Colour measurement were conducted as the method described in 3.6.

6.4.4 Ash content

The ash content was measured as described in 3.4.

6.4.5 Total carbon and protein content

The ash content was measured as described in 3.5.

6.4.6 Sample extraction

The chemical extraction was performed as described in 3.7.

6.4.7 Simulation of the *in vitro* digestion process and glycaemic glucose equivalent

The simulation of the *in vitro* digestion process and glycaemic glucose equivalent assay was carried out as described in 3.8.

6.4.8 Determination of total phenolic content

The total phenolic content was determined as described in 3.10.

6.4.9 Antioxidant activity

The antioxidant activity measurement was carried out as described in 3.12.

6.4.10 Statistical analysis

Statistical analysis was performed as described in 3.24.

6.5 Results and discussion

6.5.1 Physical characteristics and proximal compositions of cookies

The physical characteristics of dough and cookies are shown in Table 6.1. Textural properties are important factors responsible for the cookie quality. As the level of replacement of functional protein ingredients for flour increased, the textural characteristics of the cookies changed significantly ($P < 0.05$). Before baking, as the amount of functional protein ingredients increased, the hardness of dough decreased significantly, indicating that when protein ingredients were combined into dough, they were softer in comparison to the control dough. By contrast, the hardness, diameters and thickness of the experimental cookies after cooking all increased when compared with the control cookies. During the baking process, the baking loss in the experimental groups was higher compared to the control group.

The proximate composition of cookies is illustrated in Table 6.2. The moisture content in SWB cookies increased with increasing SWB addition, while the moisture content of FWB cookies decreased with increasing FWB content. The protein content of both SWB and FWB cookies

were higher than the control group ($p < 0.05$), while the C/N ratio of the experimental cookies were all lower than the control cookies.

The Pearson's correlations between the texture characteristics and proximal composition was also investigated. A negative correlation was observed between protein content and hardness of dough was observed ($R^2 = 0.633$, $p < 0.05$) (Figure 4a), revealing that the increased protein content decreased the swelling index of the starch granules and further influenced the interaction of starch and protein as well as their hydrogen bonding during the dough development, and thus resulted in the different textural properties of dough. The different water holding capacity values of protein ingredients might be another reason for the difference in texture and the moisture content of cookies. In Chapter 5, it was revealed that the water holding capacity of whey protein isolate decreased with the addition of blackcurrant concentrate and that the FWB ingredients had a higher water holding capacity than the SWB. This is in agreement with the texture results of dough and cookies in this chapter. The spread factor of a cookie is affected by the viscosity of dough and the water holding of protein ingredients in the dough to expand in volume. Higher water holding capacity results in a lower expansion since the available water in the system is not sufficient to dissolve sugar during the baking process, thus increasing the viscosity of cookies and resulting in cookies with a smaller diameter (Devi & Khatkar, 2016).

Table 6.1 Physical characteristics of dough and cookies

	Dough		Cookies		
	Dough hardness (g)	Baking loss (%)	Cookie hardness (g)	Diameters (%)	Thickness (%)
Control	756.74 ± 24.68^{a}	8.69 ± 0.18^{c}	16138.35 ± 1062.59^{c}	13.00 ± 1.35^{b}	59.8 ± 3.31^{d}
5% SWB	728.09 ± 29.37^{a}	10.10 ± 0.18^{a}	23557.55 ± 1960.01^{bc}	20.00 ± 1.11^{a}	73.30 ± 4.78^{cd}
10% SWB	530.21 ± 10.50^{d}	9.68 ± 0.17^{ab}	29911.92 ± 2615.00^{ab}	21.40 ± 1.40^{a}	98.00 ± 9.03^{bc}
15% SWB	306.28 ± 9.31^{e}	9.27 ± 0.20^{b}	30805.64 ± 785.56^{a}	21.50 ± 0.88^{a}	99.00 ± 0.20^{b}
5% FWB	666.10 ± 24.05^{b}	8.42 ± 0.43^{c}	19083.41 ± 978.87^{c}	20.60 ± 1.14^{a}	116.00 ± 9.34^{a}
10% FWB	554.80 ± 13.37^{c}	9.28 ± 0.21^{b}	25468.62 ± 1490.02^{b}	20.20 ± 1.07^{a}	84.90 ± 5.58^{c}
15% FWB	596.41 ± 22.29^{c}	9.73 ± 0.39^{ab}	23741.37 ± 2246.24^{bc}	19.90 ± 0.68^{a}	90.70 ± 10.30^{bc}

Values = Mean ± standard deviation (SD). Values within a vertical column followed by the different letter are significantly different from each other ($p < 0.05$). SWB: spray-dried whey protein isolate + blackcurrant concentrate; SWC: spray-dried whey protein isolate + imitation blackcurrant juice; FWB: freeze-dried whey protein isolate + blackcurrant concentrate; FWC: freeze-dried whey protein isolate+ imitation blackcurrant juice

Table 6.2 Proximal analysis of cookies

	Ash (%)	Moisture (%)	Protein (%)	C/N
Control	1.31 ± 0.07^{a}	8.36 ± 0.07^{b}	7.60 ± 0.24^{d}	43.97 ± 1.48^{a}
5% SWB	1.20 ± 0.05^{a}	4.11 ± 0.05^{f}	8.70 ± 0.02^{c}	36.15 ± 0.01^{a}
10% SWB	1.08 ± 0.05^{a}	7.29 ± 0.10^{e}	10.53 ± 0.01^{b}	31.41 ± 0.03^{b}
15% SWB	1.14 ± 0.03^{a}	9.79 ± 0.04^{a}	11.98 ± 0.03^{a}	28.76 ± 0.04^{c}
5% FWB	1.10 ± 0.07^{a}	8.48 ± 0.18^{b}	9.06 ± 0.04^{c}	37.00 ± 0.29^{a}
10% FWB	1.18 ± 0.02^{a}	8.19 ± 0.10^{d}	10.53 ± 0.09^{b}	32.08 ± 0.29^{b}
15% FWB	1.25± 0.05^{a}	7.60 ± 0.12^{c}	11.99 ± 0.09^{a}	27.62 ± 0.22^{c}

Means ± standard deviations (n = 3). Values in the same column with different letters differ significantly ($p < 0.05$). SWB: spray-dried whey protein isolate + blackcurrant concentrate; SWC: spray-dried whey protein isolate + imitation blackcurrant juice; FWB: freeze-dried whey protein isolate + blackcurrant concentrate; FWC: freeze-dried whey protein isolate+ imitation blackcurrant juice

In addition, negative correlations (Figure 6.4) between the C/N ratio and hardness of cookies (R^2 = 0.645, $p < 0.05$), and the C/N ratio and diameters of cookies (R^2 = 0.609, $p < 0.05$) were both obtained. These results indicate that the interaction between minerals and proteins might affect the water holding capacity of the cookie ingredients, resulting in the difference of the texture properties of the cookies (Suriya, Rajput, Reddy, Haripriya, & Bashir, 2017)

6.5.2 Colour properties

Table 6.3 shows the L^*, a^*, b^*, and ΔE values for the dough, corresponding to the surface, and ground material of the cookies. The value of L^* indicates the lightness ranging from 100 (the lightest) to 0 (the darkest). The value of a^* represents the redness (positive value) and greenness (negative value), and the value of b^* revealed the yellowness (positive value) and blueness (negative value), while the value of $\triangle E$ (colour difference) represents the colour distance within the groups. Cookies, and dough, formulated with protein ingredients were darker in colour compared with the control group (lower L^* values). Higher a^* values, and lower b^* values were found in experimental dough and cookies compared to the control groups, indicating the higher redness and the lower yellowness of experimental cookies and dough with the increasing levels of protein ingredients replacing flour. Overall, all these colour parameters were mainly influenced by the amount of protein ingredients addition. It is noticeable that ΔE value of cookies contained FWB is slightly lower than the corresponding SWB ingredients. Chapter 4 revealed that compared with the spray-drying, freeze-drying could preserve the surface anthocyanin content of blackcurrant concentrate-enriched protein ingredients more efficiently. Surface anthocyanin content is also the main factor leading to the difference in the colour parameters of cookies (Khoo, Azlan, Tang, & Lim, 2017). Therefore, the $\triangle E^*$ values of cookies containing FWB had a larger difference compared with the control group, than the cookies containing SWB.

Table 6.3 Colour profile (dough, cookies, cookie powder)

		*L**	*a**	*b**	*ΔE**
Control	Dough	66.04 ± 0.78^{a}	0.05 ± 0.18^{c}	15.01 ± 0.76^{a}	-
	Cookie	76.14 ± 0.06^{a}	-1.77 ± 0.08^{c}	25.54 ± 0.71^{a}	39.97 ± 0.49^{a}
	Cookie powder	79.98 ± 0.52^{a**}	-1.62 ± 0.06^{f}	24.01 ± 0.32^{a}	42.04 ± 0.25^{a**}
5% SWB	Dough	46.34 ± 0.85^{b}	1.97 ± 0.06^{c}	-3.70 ± 0.21^{c}	6.95 ± 0.51^{b}
	Cookie	46.75 ± 0.22^{d}	2.66 ± 0.21^{c}	7.12 ± 0.42^{c}	7.80 ± 0.32^{d}
	Cookie powder	53.51 ± 0.41^{c**}	0.66 ± 0.10^{d**}	6.15 ± 0.06^{d}	11.26 ± 0.32^{cd**}
10% SWB	Dough	39.99 ± 0.92^{b}	1.94 ± 0.09^{b}	-6.27 ± 0.78^{d}	10.71± 0.67^{cd}
	Cookie	42.27 ± 0.45^{e}	7.83 ± 0.26^{a}	6.66 ± 0.72^{c}	7.02 ± 0.36^{e}
	Cookie powder	51.15 ± 0.91^{c**}	4.52 ± 0.14^{b**}	5.37 ± 0.24^{d}	7.35 ± 0.82^{d}
15% SWB	Dough	35.23 ± 0.45^{c}	3.72 ± 0.17^{ab}	-8.29 ± 0.10^{f}	14.56 ± 0.36^{c}
	Cookie	40.76 ± 0.19^{e}	10.41 ± 0.44^{a}	6.61 ± 0.77^{c}	8.48 ± 0.71c^{d}
	Cookie powder	51.44 ± 0.51^{c**}	7.53 ± 0.06^{a**}	6.35 ± 0.13^{cd}	7.86 ± 0.38^{d}
5% FWB	Dough	47.36 ± 0.44^{b}	1.44 ± 0.12^{cd}	-1.4 ± 0.16^{b}	6.31 ± 0.17^{b}
	Cookie	58.77 ± 1.12^{b}	-0.31 ± 0.39^{d}	11.54 ± 0.38^{b}	18.26 ± 0.99^{b}
	Cookie powder	64.78 ± 0.20^{b**}	-1.05 ± 0.11^{e}	11.27 ± 0.18^{b}	23.12 ± 0.13^{b**}
10% FWB	Dough	43.44 ± 0.19^{b}	3.14 ± 0.10^{a}	-3.6 ± 0.06^{d}	6.55 ± 0.08^{cd}
	Cookie	51.01 ± 0.17^{c}	2.62 ± 0.16^{c}	8.65 ± 0.19^{c}	10.33 ± 0.27^{c}
	Cookie powder	56.52 ± 0.51^{c**}	1.02 ± 0.14^{c**}	7.52 ± 0.23^{c**}	13.89 ± 0.46^{c**}
15% FWB	Dough	38.44 ± 0.83^{b}	2.68 ± 0.14^{a}	-6.14 ± 0.26^{e}	11.26 ± 0.68^{cd}
	Cookie	47.33 ± 0.07^{d}	5.24 ± 0.15^{b}	6.76 ± 0.52^{c}	6.39 ± 0.48^{e}
	Cookie powder	52.57 ± 0.57^{c**}	3.70 ± 0.08^{b**}	6.15 ± 0.29^{cd}	9.13 ± 0.56^{cd**}

Values in the same column with different letters differ significantly ($p < 0.05$). * $p < 0.05$; ** $p < 0.01$. SWB: spray-dried whey protein isolate + blackcurrant concentrate; SWC: spray-dried whey protein isolate + imitation blackcurrant juice; FWB: freeze-dried whey protein isolate + blackcurrant concentrate; FWC: freeze-dried whey protein isolate+ imitation blackcurrant juice

6.5.3 Protein ingredients increased total phenolic content and antioxidant capacity of cookies

The total phenolic content in cookies before and after digestion are shown in Figure 6.2. The total phenolic content values of the treatment cookies were significantly ($p < 0.05$) different from the controls, and linearly increased with the increasing levels of addition of the protein ingredients in cookies. Before the digestion, SWB-enriched cookies had a higher total phenolic content compared with the corresponding FWB-enriched cookies. The highest content of total phenolic content was observed in the cookies containing 15% SWB (62.86 mg gallic aid equivalent/g), which was 3-fold higher compared with the control cookies ($p < 0.05$). After the intestinal digestion, the total phenolic content of all cookies increased dramatically compared to their undigested counterparts ($p < 0.05$). The total phenolic content in FWB-enriched cookies was higher ($p < 0.05$) than that of SWB after the digestion. 15% FWB cookies had the highest total phenolic content value of 524.92 mg gallic acid equivalent/g. These results confirm that whey protein isolate is a good carrier for delivering phenolic compounds, and exhibits a high released profile in intestinal fluids, even when the protein ingredients were prepared by different methods, in agreement with the findings by Vulić *et al.* (2019). Whey protein can be hydrolysed rapidly by different enzymes during intestinal digestion, resulting in whey protein being suitable for the controlled release of phenolics (Cirkovic Velickovic & Stanic-Vucinic, 2018). Positive correlations between protein content and total phenolic acid (before digestion) ($R^2 = 0.746$, $p < 0.05$); protein and total phenolic acid (after digestion) ($R^2 = 0.872$, $p < 0.05$) in cookies were both observed (Figure 6.4b). Therefore, the total phenolic acid significantly increased in cookies with increasing levels of protein ingredients, due mainly to the higher total phenolic acid and protein content of blackcurrant addition. In addition, the results revealed that freeze-drying might have a more positive effect on the preservation of phenolic compounds in comparison to spray-drying.

The potential health benefits of phenolic compounds result mainly from their antioxidant capacity. Thus, higher amounts of phenolic compounds in samples, can be correlated with a higher antioxidant capacity (Shahidi & Ambigaipalan, 2015). Figure 6.3 shows the antioxidant capacity of cookies by measurement with DPPH (Figure 6.3a) and FRAP assay (Figure 6.3b) and the Pearson's correlation between antioxidant capacity and total phenolic acid was also calculated. By enriching the cookie formulation using 5, 10 and 15% protein ingredients, the observed DPPH radical scavenging activities were significantly higher ($p < 0.05$) compared to the control cookies, which was consistent with the total phenolic acid results. The strongest radical scavenging ability was also found in the 15% SWB-enriched cookies (4.47 µmoL TE/g). After the intestinal digestion, the scavenging ability of the cookies increased, especially for the cookies containing the 15% protein ingredients. In line with the total phenolic acid results, 15% FWB-enriched cookies showed the strongest DPPH radical scavenging ability (9.25 µmoL TE/g), which was 2-fold higher than the undigested cookies ($p < 0.05$).

There is no single chemical assay that can accurately determine the contribution of the bioactive compounds to the total antioxidant activity of samples. Moreover, the antioxidant activity of bioactive compounds is highly influenced by the chemical transformations during the digestion (Denardin *et al.*, 2015). Therefore, the reducing antioxidant power of the cookies, measured by FRAP, was also performed. Before the digestion, 15% FWB-enriched cookies showed the highest antioxidant power (8.73 mmoL $FeSO_4$/g), followed by 15% SWB-enriched cookies (8.17 mmoL $FeSO_4$/g). The FRAP values of digested SWB-enriched cookies were higher than the corresponding undigested cookies ($p < 0.05$), while FWB-enriched cookies had decreased FRAP values after digestion. The phenolics responsible for ferric reduction may reduce or convert to certain metabolites with different chemical properties, as these polyphenols are highly sensitive to alkaline conditions. The FRAP values of SWB-enriched cookies increased after *in vitro* digestion. Spray-drying may protect these fortified

ingredients from the alkaline conditions compared to the freeze-drying. Cookies containing 15% SWB ingredients showed the strongest ferric reducing power among all cookies (9.45 mmoL $FeSO_4$/g), this was opposite to the total phenolic acid and DPPH results. With increasing levels of replacement, the FRAP values of experimental cookies increased. Positive correlations between DPPH (after digestion) and protein content of cookies (R^2 = 0.728, $p < 0.05$), and FRAP values (after digestion) and protein content (R^2 = 0.691, $p < 0.05$) were obtained and are shown in Figure 6.4b, indicating that apart from the total phenolic acid of cookies, the protein ingredients are also involved in contributing to the antioxidant ability. However, whey protein isolate could also perhaps be the embedding material for the phenolic compounds, protecting them from being degradation during the intestinal digestion (Acosta-Estrada, Gutiérrez-Uribe, & Serna-Saldívar, 2014).

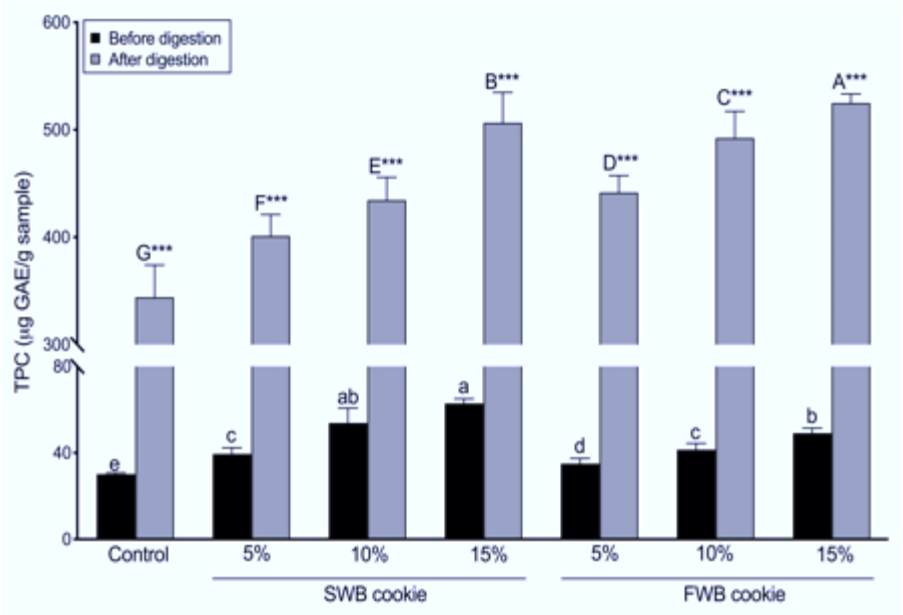

Figure 6.2 Total phenolic content (TPC) values of cookies before and after the *in vitro* digestion.

Values before the digestion with different small letters, and values after the digestion with different upper-case letters, are statistically different ($p < 0.05$). Comparison in a group is expressed by * $p < 0.05$ or ** $p < 0.01$. All values were based on dry basis. SWB: spray-dried whey protein isolate + blackcurrant concentrate; FWB: freeze-dried whey protein isolate + blackcurrant concentrate

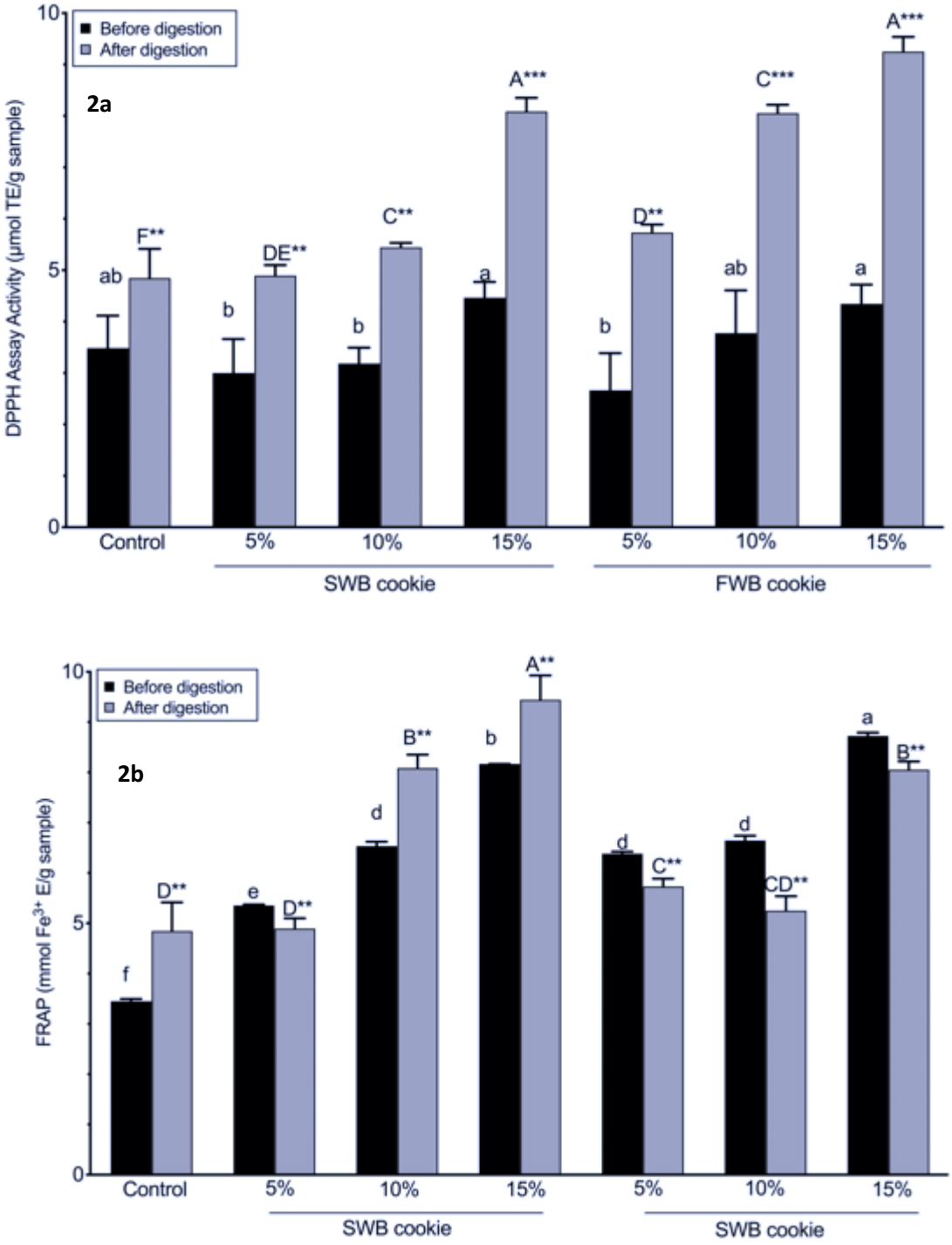

Figure 6.3 Antioxidant activity of the samples.

DPPH (2a), FRAP (2b). Values before the digestion with different small letters, and values after the digestion with different uppercase letters are statistical different ($p < 0.05$). Comparison in a group is expressed by * $p < 0.05$ or ** $p < 0.01$. All values were based on dry basis. SWB: spray-dried whey protein isolate + blackcurrant concentrate; FWB: freeze-dried whey protein isolate + blackcurrant concentrate.

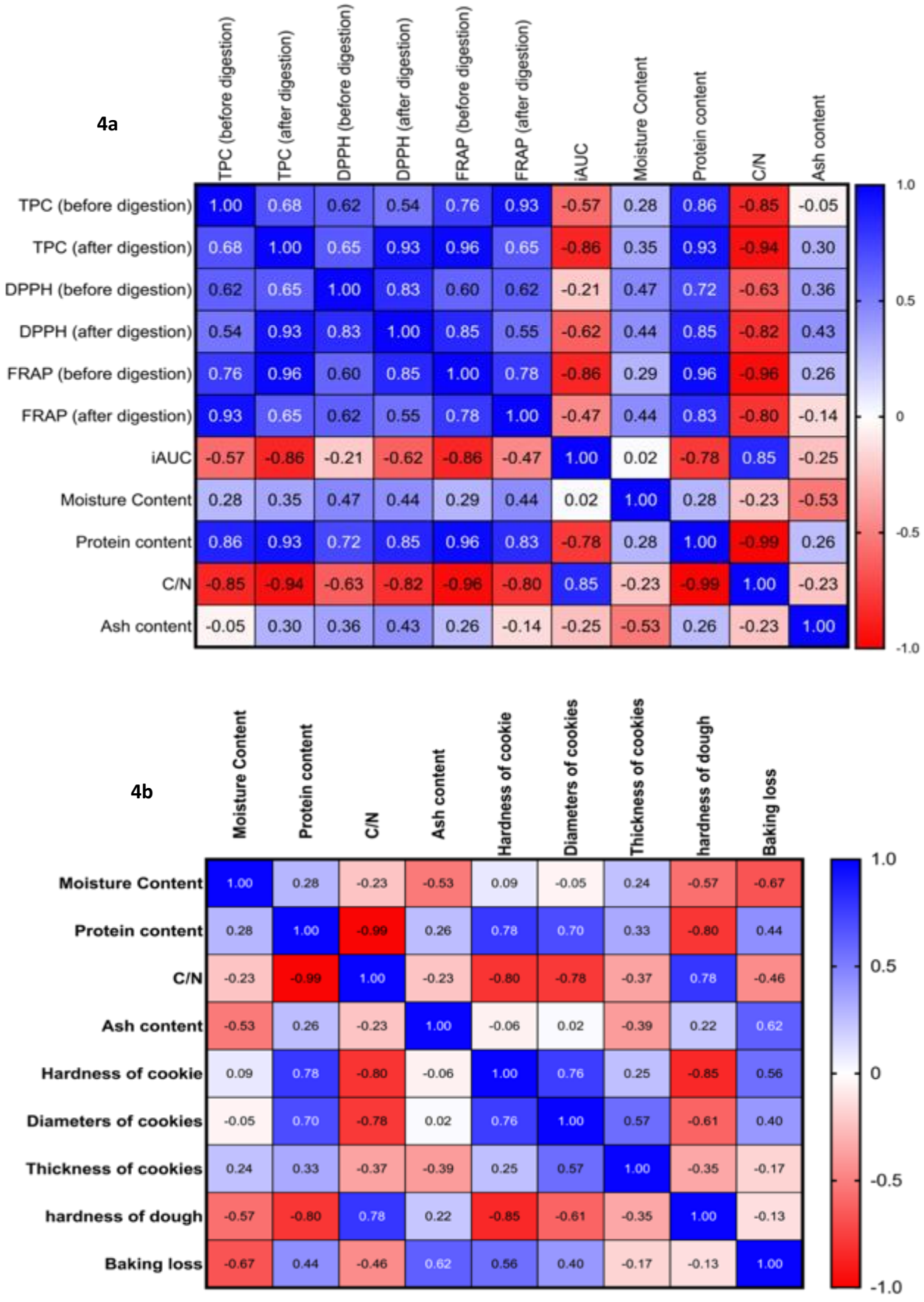

Figure 6.4 Pearson's correlations between observed values

6.5.4 Functional protein ingredients (SWB, FWB) reduced the *in vitro* glycaemic response of cookies

A glycaemic glucose equivalent assay was conducted to evaluate the reducing sugar released during a 120-min *in vitro* digestion. Figure 6.5a shows the glucose released calculated as the reducing sugar hydrolysed from starch by digestive enzymes. All cookie groups showed a sharp increase in the rate of reducing sugar released in the first 20 min of the digestion, and then during 20-120 min, the rate of reducing sugar released decreased or even flattened off. Figure 6.5b illustrates the area under curve values of cookies. The lower the area under curve values of the cookies, the lower glycaemic response the cookies had. Increasing the amount of protein ingredients in cookies reduced the sugar released and the glycaemic response significantly compared with the control cookies ($p < 0.05$). The lowest area under curve value of 394 mg glucose/g was found in cookies containing 15% FWB, which was 2.5-fold lower than the area under curve value of the control cookies. Negative correlations were observed between area under curve and total phenolic content (after digestion) ($R^2 = 0.734$, $p < 0.05$), and area under curve and protein content ($R^2 = 0.605$, $p < 0.05$). These results reveal that the bioactive compounds and protein in cookies could be responsible for the lower rate of reducing sugar released and the starch degradation of cookies. Blackcurrants contain high amounts of fibre and phenolic compounds, which are the effective inhibitors of digestive enzymes (Annunziata *et al.*, 2020). The inhibitory enzymes activities of these compounds may result from the interactions between digestive enzymes and phenolics, including hydrogen bonding, hydrophobic and ionic interactions, resulting in the formation of an “inhibitor-enzyme” or the “inhibitor-starch-enzyme” complex (Martinez-Gonzalez *et al.*, 2017). In addition, phenolics are sensitive to the alkaline conditions of intestinal digestion and produce reactive species, which can interact with the free amino groups of the digestive enzymes. These reactions can change the solubility, molecular weight, and secondary and tertiary

structures of the protein as the cookie is being digested, thus altering the way that the digestion progresses (Keppler, Schwarz, & van der Goot, 2020; Wu, Liu, Qin, Wang, & Wu, 2019). FWB-enriched cookies had a lower glycaemic response than the corresponding cookies containing SWB, further revealing that freeze-drying might be a better option for formation the model food containing bioactive compounds.

6.6 Conclusion

The chapter showed that the formulation of protein ingredients in cookies obtained relatively better texture characteristics, higher total phenolic content and DPPH radical scavenging capacity, reducing power and anti-hyperglycaemic activity compared with the normal cookies. Better results were obtained from the freeze-drying technique than the spray-drying. These findings suggest that there is a big potential for whey protein-blackcurrant concentrate encapsulates as an antioxidant, colourant, and protein enhancer in food systems, due to its high content of bioactive compounds, antioxidant capacity, and protein content. Overall, both SWB and FWB are efficient for functional food development, with improved nutrition, colour, texture and bioactive properties. The novelty of this research is the utilisation of high protein ingredients to replace flour content. Meanwhile the functionalised protein ingredients have been found with hypoglycaemic effects. These novel functional protein ingredients can also be applied in other common starchy food products, such as noodles, cakes, muffins. Future research should investigate the consumer acceptance of the functional cookie product.

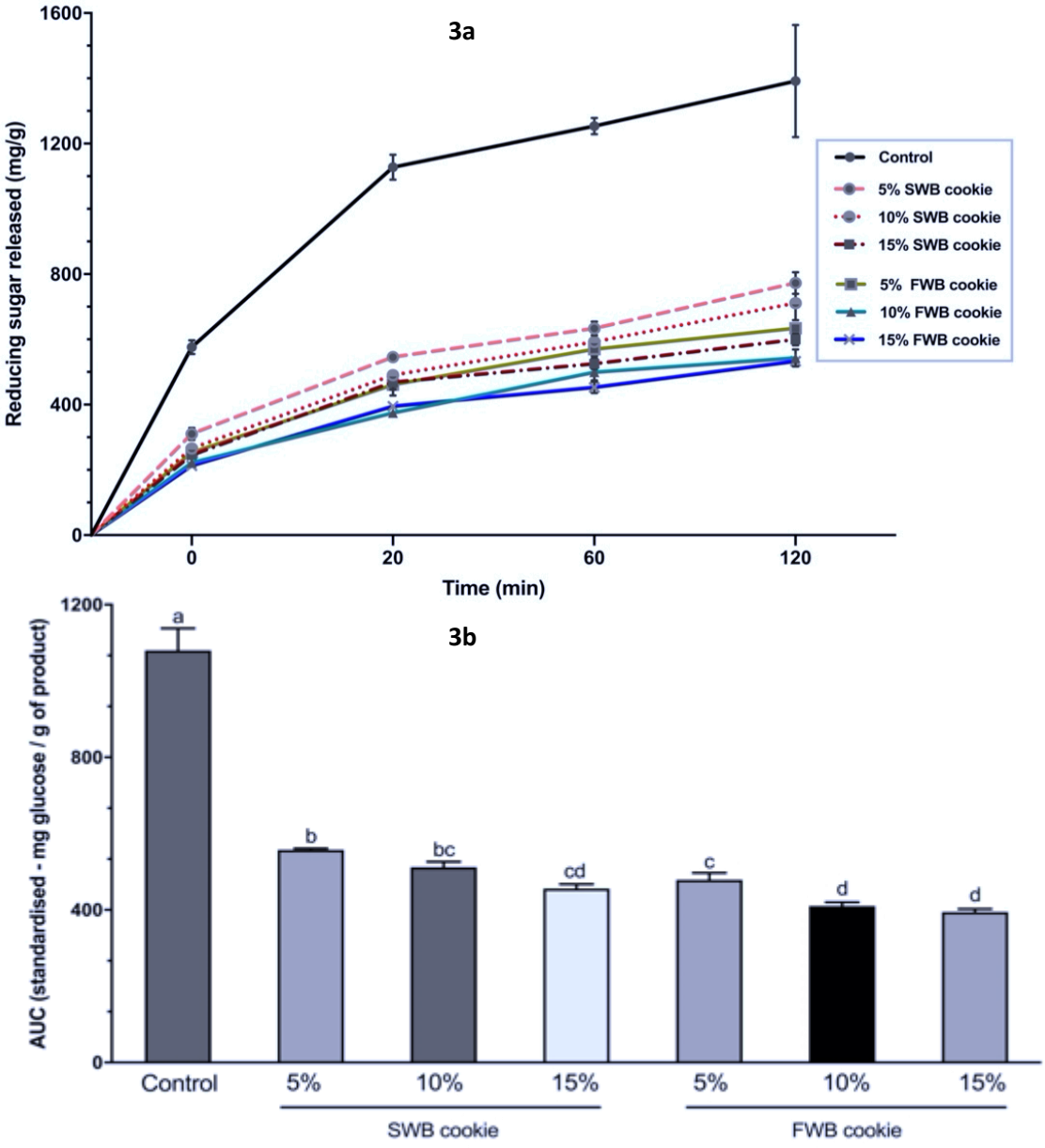

Figure 6.5 Reducing sugar release after *in vitro* digestion.

a: The reducing sugar released from cookies during a 120-min in vitro digestion; b: Area under curve (AUC) values of cookies during the in vitro digestion. Bars with different letters differ significantly ($p < 0.05$). All values were based on dry basis. SWB: spray-dried whey protein isolate + blackcurrant concentrate; FWB: freeze-dried whey protein isolate + blackcurrant concentrate.

Chapter 7

Functionalisation of sodium caseinate fortified with blackcurrant concentrate via spray-drying and freeze-drying techniques: physicochemical and nutritional properties

(Published in *LWT*, DOI: 10.1016/j.lwt.2021.111051)

7.1 Abstract

Blackcurrant anthocyanins are known to possess numerous biological functions. However, they are sensitive to light, temperature, and oxygen, and this is a limitation for their direct incorporation into food products. In contrast to a large volume of literature on the health benefits of blackcurrant compounds, there are limited studies on their practical applications in fabricated food products. This chapter examines the combination of sodium caseinate solution with blackcurrant concentrate using spray-drying and freeze-drying strategies. This created a novel functional protein ingredient which was able to prevent the degradation of sensitive biological compounds. The encapsulation efficiency using freeze-drying was more effective than spray-drying (94.57 ± 0.25 % compared to 96.36 ± 0.12 %). The proximate and colour profiles of the powders showed that these protein ingredients could be used to improve the protein content and change colour property of food products. The synergistic effects of blackcurrant polyphenols, and digested protein peptides on antioxidant activity were evaluated. These encapsulated ingredients showed on the amylase inhibitory activity and decreased reducing sugar released during an *in vitro* digestion process, illustrating their potential use as functional additions for the purpose of slowing blood sugar spiking.

7.2 Graphic abstract

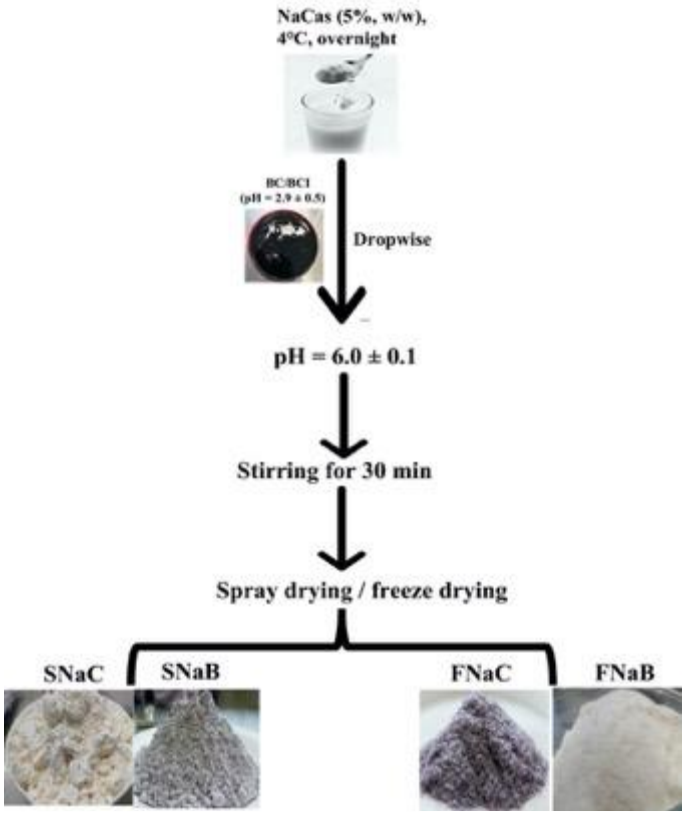

Figure 7.1 The production procedures of sodium caseinate-blackcurrant concnetrate particles.

NaCas: sodium caseinate; SNaB: spray-dried sodium caseinate + blackcurrant concentrate; SNaC: spray-dried sodium caseinate + imitation blackcurrant juice; FNaB: freeze-dried sodium caseinate + blackcurrant concentrate; FNaC: freeze-dried sodium caseinate+ imitation blackcurrant juice

7.3 Introduction

Bovine milk can provide high nutritive value for individuals (Haug *et al.*, 2007). Accounting for around 80% of total protein content of bovine milk, casein has been traditionally processed into cheese. Casein exists in the form of casein micelles, comprising alpha casein, beta casein, kappa casein (Li & Zhao, 2019). Commercially, bovine casein has been processed into sodium caseinate or calcium caseinate and used as gelling, emulsion, and foaming ingredients (Zhan *et al.*, 2018). Casein solubility is in a pH-dependent manner and is also affected by ionic strength and other chemical compositions (Sutariya, Huppertz, & Patel, 2017).

Blackcurrant fruit is abundant in vitamins, minerals, and polyphenols, especially anthocyanins (Boath *et al.*, 2012). Over 90% of blackcurrant polyphenols are anthocyanins, these include delphinidin 3-O-rutinoside, delphinidin 3-O-glucoside, cyanidin 3-O-glucoside, and cyanidin 3-

O-rutinoside (Cortez & Gonzalez de Mejia, 2019). These components are related to reported health benefits, such as eye health, gut health, antioxidant capacity, and anti-inflammatory activities (Nile & Park, 2014). However, these bioactive compounds are sensitive to light and thermal treatment. More studies are needed to understand how to overcome these challenges that astringency and bitterness present for the formulation of desirable blackcurrant concentrate products without the addition of sugar. It is equally important to develop a novelty method to address this issue without negating the health benefits of these compounds (Laaksonen *et al.*, 2014). A high protein, low-calorie, and low-fat diet with extra health benefit components, such as dietary phenolics, has become into a new trend (Seid & Rosenbaum, 2019).

Milk protein has been widely used as wall material for sensitive bioactive compounds (Livney, 2010). Spray-drying, and freeze-drying have been used as encapsulation technologies to produce encapsulates with health benefits (Rezvankhah *et al.*, 2020). Casein is a proline-rich protein which has high affinities with polyphenols (Yan *et al.*, 2009). Blackcurrant concentrate has low acidity (pH = 2.9), while sodium caseinate has a higher solubility at pH 6.0 to 8.0 than at other pH values (Khwaldia, Banon, Perez, & Desobry, 2004). Casanova *et al.* (2018) reported that sodium caseinate can be used as a carrier for cyanidin 3-O-glucoside, due to its natural binding affinity with cyanidin 3-O-glucoside. Nascimento *et al.* (2020) recently reported using casein hydrogels to carry and deliver anthocyanins from jaboticaba fruit. However, there has been no research, if any, on the utilisation of sodium caseinate as carriers for fruit polyphenols. In Chapter 4, 5, and 6, the relationship between whey protein isolate and blackcurrant phenolic compounds were investigated. In this chapter the focus is on the development of functional sodium caseinate protein ingredients in a food-compatible manner with the health benefits of blackcurrant via spray- and freeze-drying strategies. The chemical composition, and colour properties of the encapsulated particles were measured. Total

phenolic content and antioxidant capacity before and after *in vitro* digestion were determined together with the reducing sugar released. Encapsulation efficiency of four main anthocyanins, as well as IC_{50} value of these encapsulated particles towards α-amylase inhibitory activity was conducted.

7.4 Methods

7.4.1 Sample preparation

Protein ingredients (SNaC, SNaB, FNaC, FNaB) were prepared as described in 3.1.

7.4.2 Ash content

The ash content was measured as described in 3.4.

7.4.3 Total carbon and protein content

The ash content was measured as described in 3.5.

7.4.4 Colour measurement

The colour profiles of the samples were recorded by following the methods described in 3.6.

7.4.5 Sample extraction

The chemical extraction was performed as described in 3.7.

7.4.6 Simulation of *in vitro* digestion process and glycaemic glucose equivalent

The simulation of *in vitro* digestion process and glycaemic glucose equivalent assay was carried out as described in 3.8.

7.4.7 Determination of total phenolic content

The total phenolic content was determined as described in 3.10.

7.4.8 Determination of total anthocyanin content, surface anthocyanin content, and encapsulation efficiency

The total anthocyanin content, surface anthocyanin content, and encapsulation efficiency were determined as described in 3.11.

7.4.9 Antioxidant activity

The antioxidant activity measurement was carried out as described in 3.12.

7.4.10 Alpha-amylase inhibition assay

Alpha-amylase inhibition assay was conducted as described in 3.13.

7.4.11 Statistical analysis

Statistical analysis was carried out as described in 3.24.

7.5 Results and discussion

7.5.1 Component analysis

Table 7.1 presents the ash, the moisture, and the protein content of powders. The addition of blackcurrant concentrate significantly increased the ash content of sodium caseinate particles ($p < 0.05$). Blackcurrant fruits are rich in potassium, calcium, magnesium, which play a vital role in biological processes (Kahu, Jänes, Luik, & Klaas, 2009). Ash content of a food product is a direct reflection of mineral content within a food. The addition of blackcurrant concentrate increased the mineral content of sodium caseinate particles. Freeze-dried sodium caseinate particles had lower moisture content (ranging from 2.59% to 4.31%) than spray-dried sodium caseinate particles (ranging from 4.66% to 5.02%) ($p < 0.05$). The obtained higher moisture content of spray-dried particle may be due to its smaller particle size and larger surface area than freeze-dried particle, leading to its hygroscopicity property. These results were in agreement with a previous study from Correia *et al.* (2017), reporting that spray-dried protein particles had a higher moisture content than freeze-dried protein particles. In addition, the

moisture content is an indicator of product stability. It was observed that the freeze-dried particles with lower moisture content had a better stability than spray-dried protein particles. Although the addition of blackcurrant concentrate decreased by approximately 3% protein content of these sodium caseinate particles ($p < 0.05$), all these sodium caseinate particles contain more than 80% protein. Potentially, these particles could be utilised as nutritional ingredients to increase protein content of starchy food, thus reducing the glycaemic response.

Table 7.1 Proximate analysis

	Ash (%)	Moisture (%)	Protein (%)	C/N
SNaC	3.88 ± 0.07^{b}	5.02 ± 0.06^{a}	87.11 ± 0.30^{a}	3.84 ± 0.01^{a}
SNaB	4.08 ± 0.06^{ab}	4.66 ± 0.10^{bc}	84.54 ± 0.27^{b}	3.74 ± 0.01^{b}
FNaC	3.86 ± 0.06^{ab}	2.59 ± 0.15^{bc}	87.62 ± 0.18^{a}	3.81 ± 0.01^{a}
FNaB	4.11 ± 0.02^{a}	4.31 ± 0.18^{ac}	84.70 ± 0.25^{b}	3.71 ± 0.01^{b}

Means ± standard deviations (n = 3). Values in the same column with different letters differ significantly (p < 0.05). All values are based on dry basis. SNaB: spray-dried sodium caseinate + blackcurrant concentrate; SNaC: spray-dried sodium caseinate + imitation blackcurrant juice; FNaB: freeze-dried sodium caseinate + blackcurrant concentrate; FNaC: freeze-dried sodium caseinate+ imitation blackcurrant juice

Table 7.2 Colour analysis

Group	*L**	*a**	*b**	*⊿E*
SNaC	96.60 ± 0.52^{a}	-0.60 ± 0.08^{d}	3.62 ± 0.16^{b}	51.30 ± 0.52^{a}
SNaB	80.50 ± 0.06^{b}	5.07 ± 0.02^{b}	-3.25 ± 0.2^{c}	34.70 ± 0.06^{c}
FNaC	89.5 ± 0.43^{c}	-1.86 ± 0.06^{c}	10.01 ± 0.09^{a}	45.40 ± 0.40^{b}
FNaB	55.3 ± 0.70^{d}	7.69 ± 0.03^{a}	-6.2 ± 0.123^{d}	11.60 ± 0.54^{d}

Mean ± standard deviation. Means with different letters within the same column are statistical different (p < 0.05). SNaB: spray-dried sodium caseinate + blackcurrant concentrate; SNaC: spray-drie sodium caseinate + imitation blackcurrant juice; FNaB: freeze-dried sodium caseinate + blackcurra concentrate; FNaC: freeze-dried sodium caseinate+ imitation blackcurrant juice

7.5.2 Colour properties

The colour parameter is important for further application of the protein ingredients. Table 7.2 illustrates the L^*, a^*, b^*, and ΔE values for spray-dried and freeze-dried protein particles. Drying methods had a significant effect on all the colour parameters of protein particles. The freeze-dried samples had a deeper red colour with significantly higher ($p < 0.05$) a^* value than the spray-dried samples. Both spray-dried and freeze-dried samples had a significant increase in redness, which could be attributed to the dyeing effect of anthocyanins (Khoo *et al.*, 2017). Potentially, these particles could be utilised as novel high protein ingredients with natural food coloration properties.

7.5.3 Total phenolic content and antioxidant activity

Figure 7.2a illustrates the total phenolic content values of sample extracts and hydrolysates as detected via the Folin-Ciocalteu method. Total phenolic content values of both SNaC and FNaC extracts were comparatively high, even without the addition of blackcurrant concentrate. This may due to the interference from other components existing in the extracts (Granger, Gallagher, Fuerst, & Alldredge, 2011), such as calcium from caseinate, ascorbic acid and sugars from imitation blackcurrant concentrate (Prior, Wu, & Schaich, 2005). The total phenolic content values of SNaB and FNaB were significantly higher than their corresponding controls (SNaC and FNaC) ($p < 0.05$). This could be attributed to the addition of blackcurrant concentrate. Both SNaB and FNaB exhibited a significant decline ($p < 0.05$) in their total phenolic content values after the stimulated *in vitro* digestion process, which may be due to the partial degradation of total phenolic content components. It is worth noting that during the process of measuring total phenolic content in the samples, a cloudy formation was observed in the test tube after adding 7.5% Na_2CO_3 into the mixture containing 0.1mol/L Folin-Ciocalteu reagent. (Vázquez *et al.*, 2015). A potential explanation for this phenomenon is that the solubility of casein components and hydrolysates is pH dependent (Rajarathnam,

Nongonierma, O'Sullivan, Flynn, & FitzGerald, 2016; Strange, Van Hekken, & Holsinger, 1994). After the addition of 7.5% Na_2CO_3 into the test tube, the pH value of the mixture containing sodium caseinate increased, and the soluble casein components and hydrolysates formed the flocculation. Hence, the total phenolic content in samples couldn't be quantified accurately, and it may be underestimated.

Figure 7.2b and 7.2c illustrate the antioxidant capacity of sample extracts and hydrolysates. There was no significant difference between sample extracts, which can be attributed to the encapsulation effects of sodium caseinate on bioactive molecules. The digestion process could increase the antioxidant capacity of the blackcurrant concentrate-containing protein powder by liberating the bioactive peptides, some of which were reported to have potential antioxidant capacity (Suarez-Jimenez, Burgos-Hernandez, & Ezquerra-Brauer, 2012). In this chapter, the higher antioxidant capacity of both SNaB and FNaB's hydrolysates can be attributed to a synergism of radical scavenging activity of released peptides and phenolic compounds (Jiang *et al.*, 2018). Lang *et al.* (2021) found that α-casein and β-casein protected the antioxidant capacity of blueberry anthocyanins and increased the bioaccessibility of blueberry anthocyanins during intestinal digestion. However, FNaB had a significant lower FRAP value ($p < 0.05$) than that of SNaB, which can be explained by different bioactive compounds release mechanisms of spray-dried particles and freeze-dried particles (Ezhilarasi, Karthik, Chhanwal, & Anandharamakrishnan, 2013)

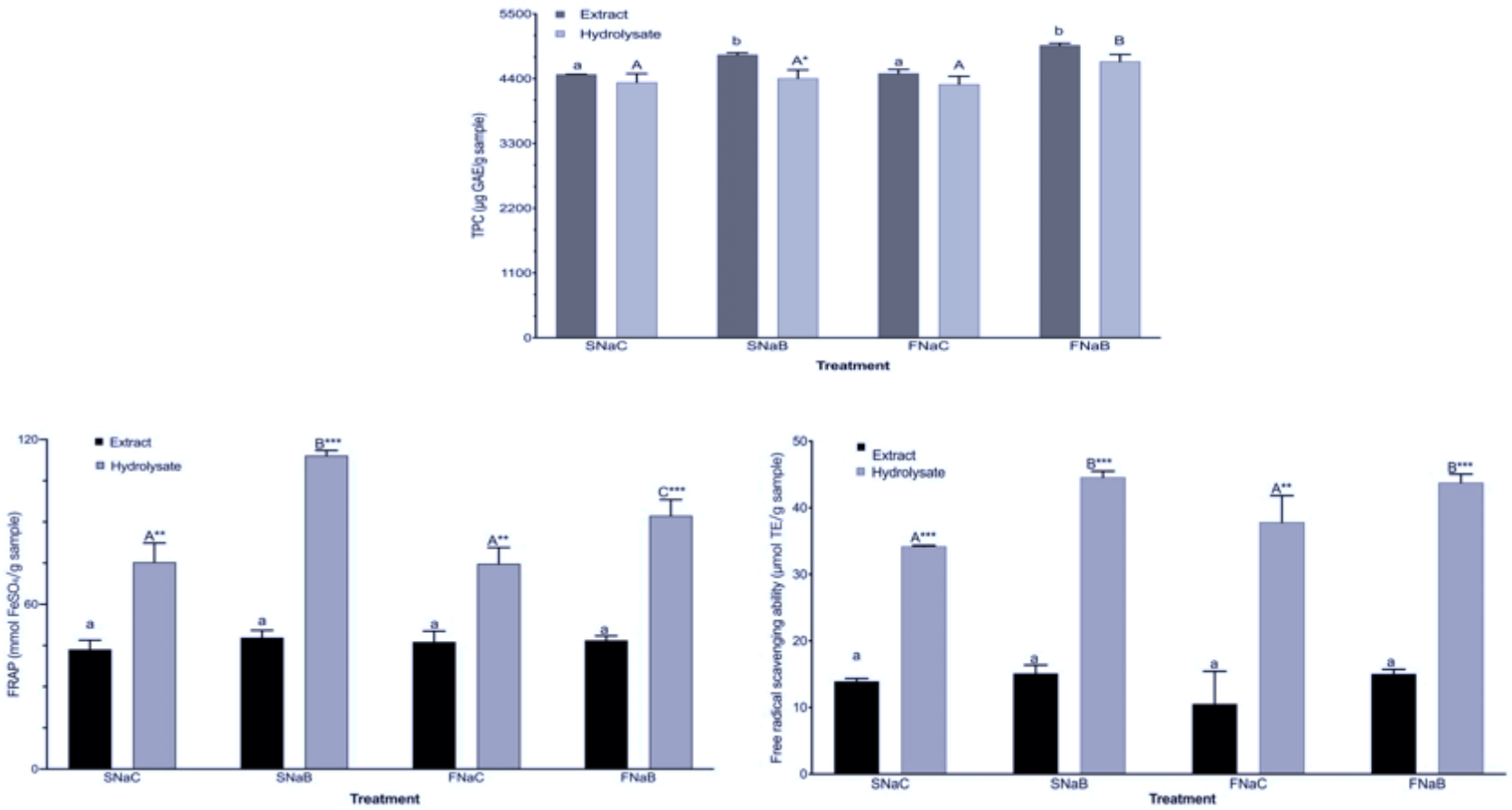

Figure 7.2 Comparison of total phenolic content (TPC) (2a) and antioxidant capacity (2b. FRAP assay; 2c. DPPH assay) of sample extracts and hydrolysates.

The columns stand for mean values. Error bars indicate standard deviation (n = 3). Comparison among extracts is expressed by different lowercase letters, and within hydrolysates is expressed by different capital letters, while before and after digestion is indicated by the asterisks ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$), and different letters indicate samples that are significantly different from each other ($p \leq 0.05$).

7.5.4 Individual anthocyanin content and surface anthocyanin content in extracts and the encapsulation efficiency

Table 7.3 presents the content of anthocyanins and the corresponding surface anthocyanins in the extracts from encapsulated powders. Four anthocyanins, including delphinidin 3-O-rutinoside, delphinidin 3-O-glucoside, cyanidin 3-O-glucoside, and cyanidin 3-O-rutinoside were detected. The extract of FNaB had higher contents of anthocyanins than SNaB ($p < 0.05$). Among these four anthocyanins, cyanidin 3-O-rutinoside accounted for the highest amount in the extracts of both SNaB and FNaB, with values 8840.54 ± 119.60 μg/100 g, and 10361.82 ± 188.47 μg/100 g, respectively. Regarding the content of surface anthocyanins in the extracts, the percentage of surface anthocyanins content in the SNaB extract ranged from 5.20 ± 0.01% to 6.35 ± 0.11%, while it varied from 3.19 ± 0.09% to 7.54 ± 0.10% in FNaB extract. Cyanidin 3-O-rutinoside obtained the least percentage of surface anthocyanins content among all anthocyanins ($p < 0.05$), both in the extracts of SNaB (5.20 ± 0.01%) and FNaB (3.19 ± 0.09%). The percentage of the surface delphinidin 3-O-glucoside, delphinidin 3-O-rutinoside, and cyanidin 3-O-rutinoside content in SNaB was higher than those in FNaB ($p < 0.05$), while FNaB extract amounted to more surface cyanidin 3-O-glucoside content of the total than SNaB extract ($p < 0.05$). Table 7.3 shows the total anthocyanin content in the extracts. theFNaB extract (20076.30 ± 212.24 μg/100 g) had significantly higher total anthocyanin content than the SNaB extract (17236.25 ± 31.17 μg/100 g) ($p < 0.05$). The encapsulation efficiency of spray-drying and freeze-drying were 94.57 ± 0.25 %, and 96.36 ± 0.12% respectively. These results suggest that freeze-drying can preserve the anthocyanins more effectively than spray-drying. Spray-drying and freeze-drying are widely used encapsulation strategies with their unique encapsulation mechanisms, leading to a totally different particle morphology (Laokuldilok & Kanha, 2015). encapsulation efficiency of these particles was frequently investigated, which depends on many factors, such as atomisation parameters and inlet/outlet air temperature of

spray-drying, the ratio of macromolecules and small molecules of solution (Tontul & Topuz, 2017). Herein, freeze-drying presented better encapsulation efficiency than spray-drying, which can be inferred to thermally sensitivity of anthocyanins during spray-drying and/or the unsaturated state of blackcurrant anthocyanins to proteins molecules (Laokuldilok & Kanha, 2015).

7.5.5 Alpha-amylase inhibition activity

Alpha-amylase is an important enzyme in the body that hydrolyses starch and glycogen into glucose and maltose, which are readily absorbed by human body. Interference with this enzyme activity is regarded as a potential manner to modulate sugar absorption. Figure 7.3 illustrates the IC_{50} values of the extracts towards α-amylase. As the positive control, acarbose, a purified synthetic α-amylase inhibitor, was possessing the strongest α-amylase inhibitory activity (IC_{50} value = 65.32 ± 0.96 μg/mL) (Nam *et al.*, 2009). Both the SNaB and FNaB ingredients had much lower ($p < 0.05$) IC_{50} values (IC_{50} of SNaB = 123.26 ± 11.49 μg/mL, IC_{50} of FNaB = 108 ± 14.03 μg/mL) than the corresponding control groups (IC_{50} of SNaB = 174.53 ± 6.78 μg/mL, IC_{50} of FNaB = 214.78 ± 13.28 μg/mL), revealing that the addition of blackcurrant concentrate improved the α-amylase inhibitory activity of the sodium caseinate ingredients. Due to its high polyphenol content, blackcurrant has been considered to have alpha-amylase inhibitory activity (Castro-Acosta *et al.*, 2017). Specifically, a previous study (Hui *et al.*, 2020) has found that this α-amylase inhibitory activity of blackcurrant was generated from the combining effects of blackcurrant anthocyanins on enzyme active sites. In addition, it was not surprising to find that FNaB exhibited stronger inhibition activity than SNaB ($p < 0.05$), due mainly to FNaB exposed to a relatively lower processing temperature than SNaB. Laokuldilok and Kanha (2015) reported that freeze-drying process prevented the adverse effect of high temperature on thermally sensitive anthocyanins and capture a higher content of anthocyanins. Within the freeze-dried microcapsules, large amount of anthocyanins were well

preserved. It was compatible with the current report, demonstrating that FWB had a higher surface anthocyanins content compared to SWB. Therefore, we conclude that freeze-drying coul f anth

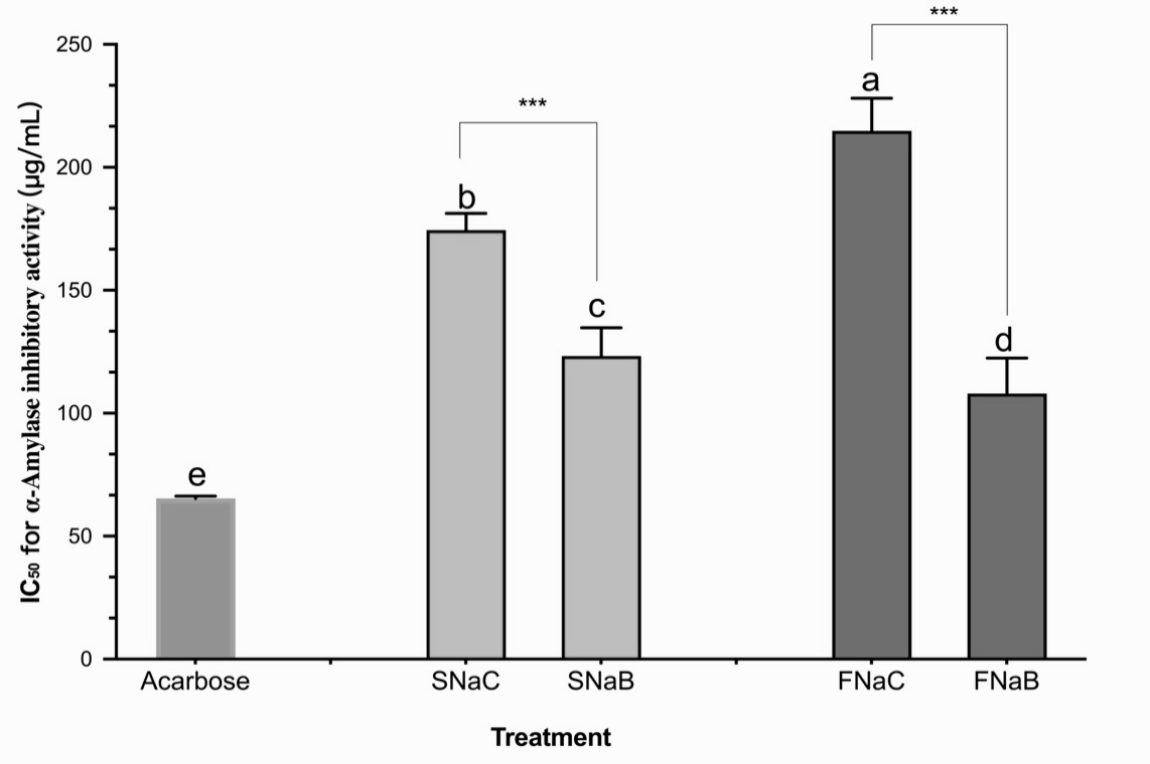

Figure 7.3 Alpha-amylase inhibitory activity of sample extract.

The columns stand for mean values. Error bars indicate standard deviation (n = 3). Different letters indicate samples that are significantly different from each other. Significant difference between spray-dried and freeze-dried samples is indicated by the asterisks ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). SNaB: spray-dried sodium caseinate + blackcurrant concentrate; SNaC: spray-dried sodium caseinate + imitation blackcurrant juice; FNaB: freeze-dried sodium caseinate + blackcurrant concentrate; FNaC: freeze-dried sodium caseinate+ imitation blackcurrant juice.

Table 7.3 Total and surface anthocyanin content of blackcurrant concentrate anthocyanins by spray-drying and freeze-drying

	TAC (µg/100g)		SAC (µg/100g)	
Anthocyanins	Spray-drying	Freeze-drying	Spray-drying	Freeze-drying
D3G	2103.31 ± 96.81	2424.06 ± 84.96***	114.99 ± 5.96	85.79 ± 3.23***
D3R	1547.15 ± 43.07	1783.25 ± 50.22***	84.91 ± 4.11	65.25 ± 2.04***
C3G	836.41 ± 21.13	981.31 ± 24.54***	53.09 ± 2.29	74.01 ± 2.49***
C3R	8840.54 ± 119.6	10361.82 ± 188.47***	459.81 ± 19.70	330.42 ± 17.57***
TAC	*17236.25 ± 31.17*	*20076.30 ± 212.24****	*936.72 ± 43.46*	*730.83 ± 26.46****

*Means ± standard deviations (n = 3). Significant difference between spray-drying and freeze-drying is indicated by the asterisks (*p < 0.05, **p < 0.01, ***p < 0.001). TAC: total anthocyanin content; SAC: surface anthocyanin content; D3R: Delphinidin 3-O-rutinoside; D3G: Delphinidin 3-O-glucoside; C3G: Cyanidin 3-O-glucoside; C3R: Cyanidin 3-O-rutinoside. SNaB: spray-dried sodium caseinate + blackcurrant concentrate; SNaC: spray-dried sodium caseinate + imitation blackcurrant juice; FNaB: freeze-dried sodium caseinate + blackcurrant concentrate; FNaC: freeze-dried sodium caseinate+ imitation blackcurrant juice*

7.5.6 Predictive *in vitro* glycaemic response

In vitro digestion experiment was carried out to reflect the reducing sugar released during the stimulated digestion process. Figure 4b demonstrates the amount of reducing sugar released over a 120-min digestion process. There was a significant decrease ($p < 0.05$) in reducing sugar released from both SNaB and FNaB comparing with their corresponding controls. Spray-drying and freeze-drying had no significant effects on the area under curve values (Figure 7.4a). blackcurrant concentrate had a high content of available carbohydrates, including starch. The decreased reducing sugar can be attributed to the α-amylase inhibition activity of anthocyanins in blackcurrant concentrate. Both SNaC and FNaC have a comparative amount of sugar based on the C/N results. As the digestion process went on, the encapsulated sugar gradually released (Barrett *et al.*, 2018). This could be an explanation for the increased reducing sugar in control groups (SNaC and FNaC) (Wang, Bao, & Chen, 2013)

7.6 Conclusion

Sodium caseinate preserved the main bioactive components of blackcurrant concentrate via spray-drying and freeze-drying, and further protected them from degradation when passing through stimulated digestive condition. The resulted particles were high in protein content (> 80%) and abundant in anthocyanins, which are positive attributes for the improvement of protein nutrition and antioxidants supplements. Such ingredients might be used as a functional addition for postprandial blood sugar regulation due to their α-amylase inhibition activity. Both spray-drying and freeze-drying could be a potential option in producing these particles. These novel protein particles have different morphologies and functional properties all of which have been studied in Chapter 8.

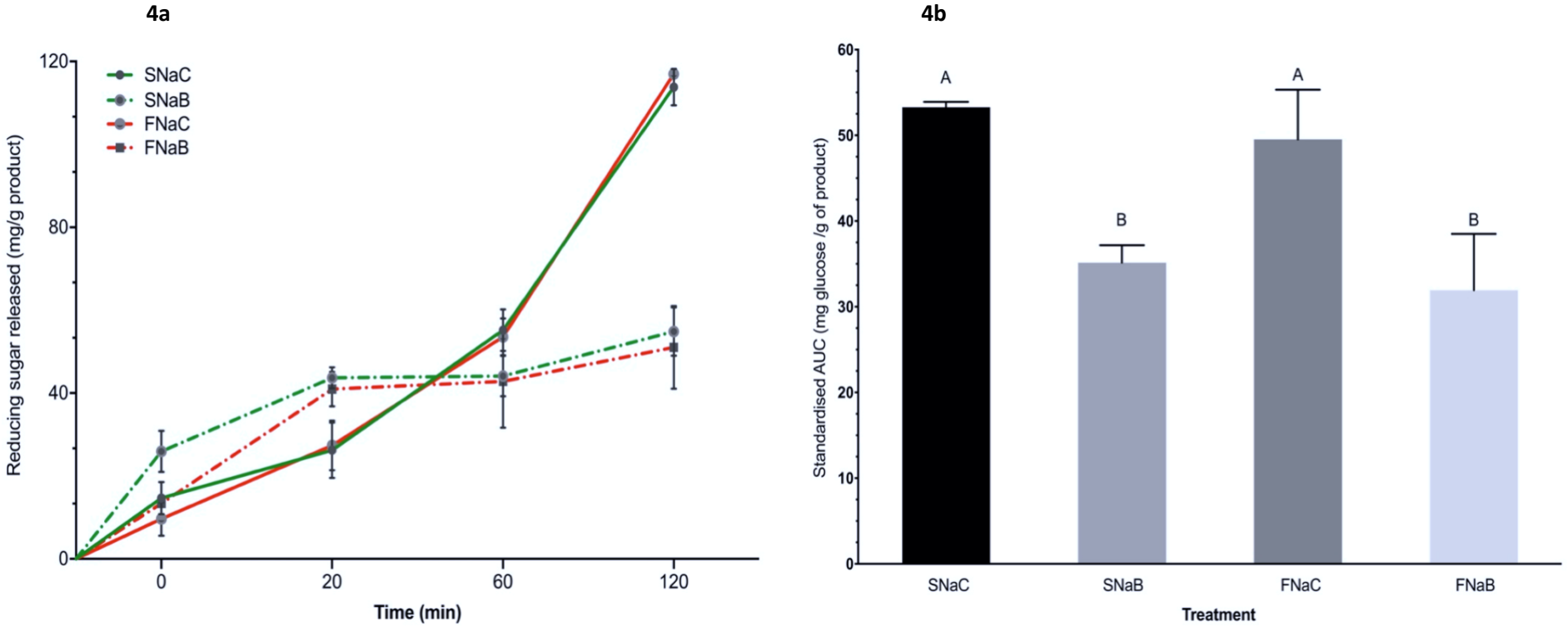

Figure 7.4 Reducing surgar released during the *in vitro* digestion.

The reducing sugar released during a 120-min in vitro digestion; Figure 4b. The area under curve (AUC) values during the in vitro digestion. Error bars indicate standard deviation (n = 3). Bars with different letters differ significantly ($p < 0.05$). NaCas: sodium caseinate; SNaB: spray-dried sodium caseinate + blackcurrant concentrate; SNaC: spray-dried sodium caseinate + imitation blackcurrant juice; FNaB: freeze-dried sodium caseinate + blackcurrant concentrate; FNaC: freeze-dried sodium caseinate+ imitation blackcurrant juice

Chapter 8

Combination of rehydrated sodium caseinate aqueous solution with blackcurrant concentrate and the formation of encapsulates via spray-drying and freeze-drying: alteration to the physical and functional properties of protein

(Published in *Journal of Food Processing and Preservation*, DOI: 10.1111/jfpp.15406)

8.1 Abstract

The practical application of protein ingredients in the preservation of bioactive compounds are of research interest. In this chapter, the aqueous solution of sodium caseinate was combined with blackcurrant concentrate to produce novel protein ingredients by spray-drying and freeze-drying. The high performance liquid chromatography results showed that both spray-drying and freeze-drying strategies effectively delivered the anthocyanins of blackcurrant concentrate. The physical and functional properties of sodium caseinate ingredients were altered by both drying strategies and the addition of blackcurrant concentrate. Drying process had an influence on shapes and sizes of the particles, and ultimately altered the rehydration properties of these protein ingredients. The inclusion of blackcurrant concentrate for sodium caseinate ingredients, and the spray-and freeze-drying strategies altered the physical properties (particle size distribution, bulk density, and morphology), functional properties (water holding capacity, oil holding capacity, foamability, and foam stability), and nutritional values of sodium caseinate ingredients, providing newly options for the development and innovation of food products.

8.2 Graphic abstract

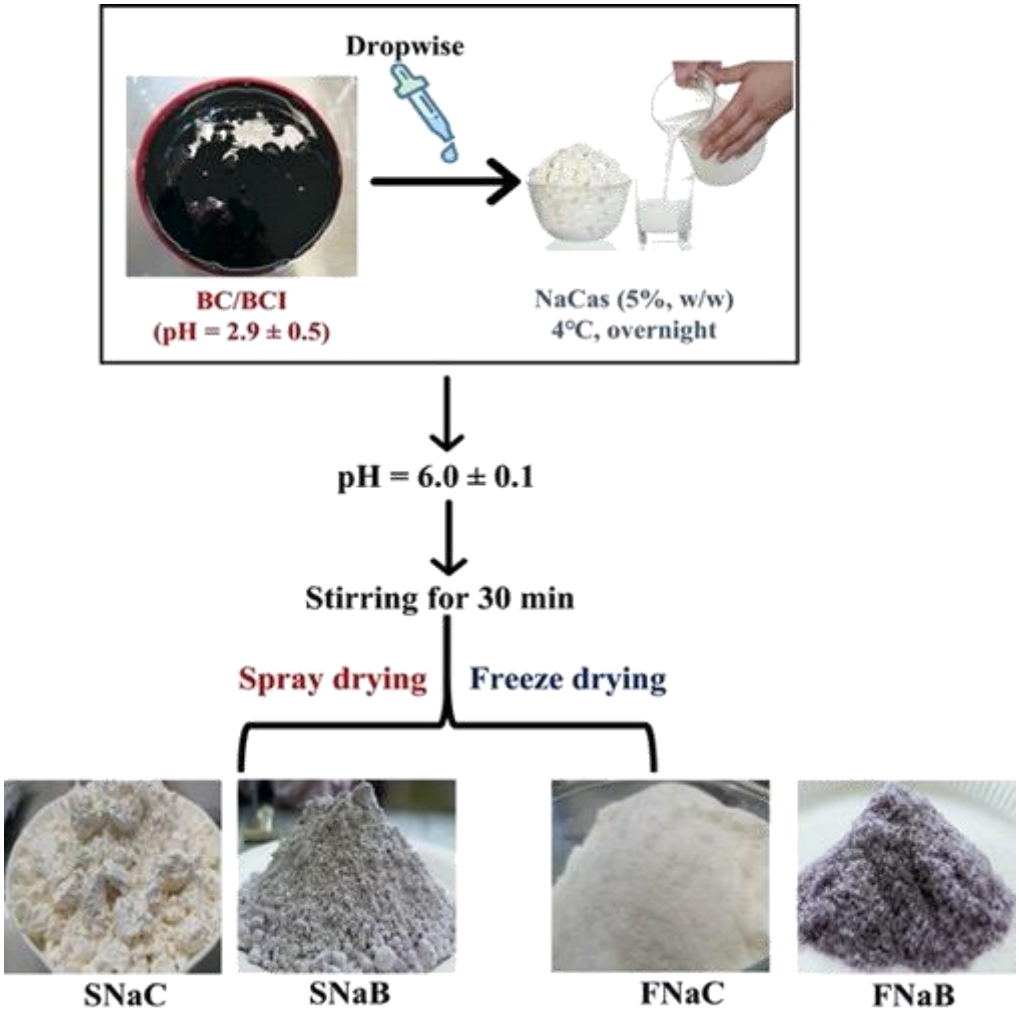

Figure 8.1 Manufacturing procedures of sodium caseinate-blackcurrant concentrate ingredients by spray-drying and freeze-drying strategies.

8.3 Introduction

Casein makes up approximately 80% of the total milk proteins, and serves as an important protein ingredient in food industry (Ranadheera *et al.*, 2016). Sodium caseinate, made from acid casein, is one the most commercially available casein-based products (Luo *et al.*, 2015). It has been used as a protein ingredient in various food products, such as bakery, yogurt, confectionery, and beverages, due to its functional and nutritional properties (Badem & Uçar, 2017). Functional properties, such as foaming, thickening, and emulsification, are critical factors to be taken into consideration when developing food formulation (Ranadheera *et al.*,

2016). However, some of the functional properties are not preferable under some specific conditions, such excessive foaming in beverage production, hardening of bars (Zhan *et al.*, 2018).

Blackcurrant is a widely planted woody shrub grown for its fruit. Blackcurrant fruit is rich in minerals, vitamins, and polyphenols, which are recognised as essential components for health benefits, such as reduction of oxidative stress, protection against cancers, cardiovascular and neurodegenerative diseases (Landbo & Meyer, 2004). The polyphenol content of blackcurrant is top among berry fruits. Anthocyanins, including delphinidin 3-O-rutinoside, delphinidin 3-O-glucoside, cyanidin 3-O-glucoside, and cyanidin 3-O-rutinoside, are accounting for more than 90% of total blackcurrant anthocyanins (Kapasakalidis, Rastall, & Gordon, 2006). Fresh blackcurrant fruit has a lemon-like sour taste, thus the fresh berry fruit is processed into juice concentrate. However, the free anthocyanins are found to be unstable during storage, processing, and digestion, thus further effecting their bioaccessibility and bioavailability (Braga, Murador, de Souza Mesquita, & de Rosso, 2018). Besides, the blackcurrant concentrate has an acidulous pH value of 2.9, which is supposed to be a challenge for its incorporation in food products (Augustin & Sanguansri, 2015).

Spray-drying and freeze-drying has been utilised as encapsulation strategies for sensitive components (Kori *et al.*, 2020). Spray-drying process involves the atomisation of the feed solution into small droplets using a two-fluid atomiser powered by a compressed air supply. The droplets are exposed to a flow of hot air. As these small droplets have a large surface area, and thus water evaporation takes place almost instantaneously within the short residence time of the drier. The droplets are transformed into dry powder particles, and then separated from the air using a cyclone (Ş. A. Milea *et al.*, 2020). Freeze-drying process involves freezing, primary drying (sublimation) and secondary drying (desorption). Typically, the feed solutions

are first frozen and water is removed from the frozen product by direct sublimation of ice into water vapour under reduced pressure (Rajam & Anandharamakrishnan, 2015).

The protective effective and delivery of protein to polyphenols have been studied widely (Ranadheera *et al.*, 2016). Luo *et al.* (2015) developed casein/pectin nanocomplexes as a potential oral delivery system for rutin. Lang *et al.* (2019) stated that casein protected blueberry anthocyanins through common processing conditions. The authors also reported that casein has the potential to protect the anthocyanins through human digestive system (Lang *et al.*, 2021).

A potential solution to the aforementioned challenges is the development of a sodium caseinate-based blackcurrant concentrate delivery system for the health benefits of blackcurrant. Meanwhile, creation a novel sodium caseinate protein ingredient with altered physical and functional properties, and nutritional quality, which providing extra options for food formulations. The aims of this chapter are to encapsulate blackcurrant concentrate by spray-drying and freeze-drying strategies using sodium caseinate as wall materials, and then further investigate the alteration on their physicochemical and functional properties.

8.4 Methods

8.4.1 Preparation of the protein ingredients

The ingredients preparation was following the method described in 3.1.

8.4.2 High performance liquid chromatography analysis of the sample extract

High performance liquid chromatography analysis was conducted as the method described in 3.9.

8.4.3 Bulk density deamination

The bulk density of the powdered ingredients was measured according to the method described in 3.17.

8.4.4 Particle sizes distribution

The particle sizes were measured by dynamic light scattering as described in 3.18.

8.4.5 Morphology properties

The surface morphology of the particles was observed by scanning electron microscopy following the protocol described in 3.16.

8.4.6 Foamability, Foam stability, and microscope structure of the foam

Foam formation capacity, foam stability, and microscope structure were following the methods described in 3.14.

8.4.7 Water holding capacity and oil holding capacity

Water holding capacity and oil holding capacity were measured as the methods described in 3.15.

8.4.8 Statistical analysis

Statistical analysis was performed as described in 3.24.

8.5 Results and discussion

8.5.1 High performance liquid chromatography analysis

Cyanidin and delphinidin, considered the most abundant anthocyanins in blackcurrant, is known to interact with protein molecules and numerous bio-functionalities (Bishayee *et al.*, 2010). As shown in Figure 8.2, cyanidin and delphinidin were identified in SNaB, with respective value as 82.55 ± 1.04 and 71.39 ± 1.07 μg/g. However, in FNaB, the content of cyanidin and delphinidin was 59.55 ± 0.54 and 53.61 ± 0.13 μg/g, respectively. These results confirmed the stability and effective delivery of blackcurrant anthocyanins by SNaB and FNaB. Moreover, due to the higher content of anthocyanins detected in spray-dried particles, spray-drying seems to be a more appropriate encapsulation manner for blackcurrant concentrate

compared to freeze-drying. This observation was consistent with a previous study that spray-dried particles retained more bioactive components (Wu, Hui, Stipkovits*, et al.*, 2021). Papoutsis *et al.* (2018) have compared the encapsulation efficacy of spray-drying and freeze-drying, and conclude that freeze-drying has higher encapsulation efficacy. The difference in the content of blackcurrant anthocyanins retention might be attributed to its unique particle properties of SNaB and FNaB, such as particle size, shape, and surface morphology, which could lead to the different released mechanisms during the extraction procedure.

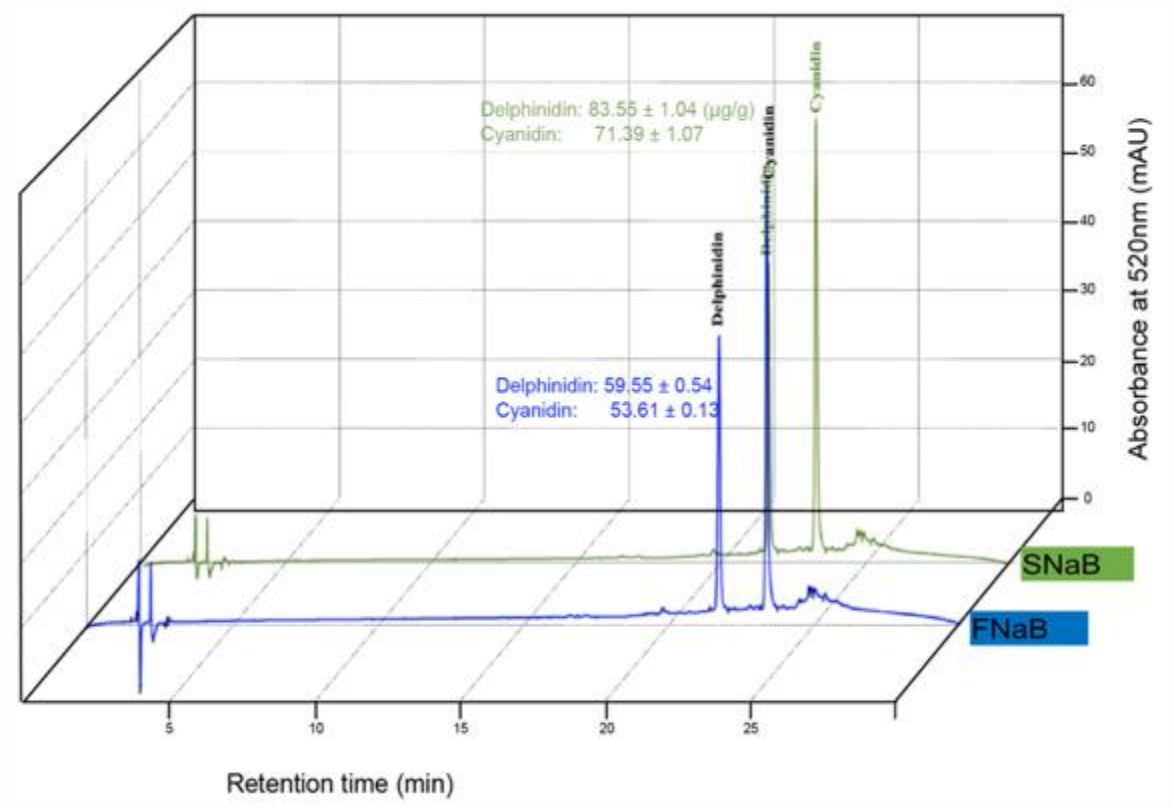

Figure 8.2 Concentration of cyanidin and delphinidin in functional ingredients (SNaB, FNaB) extracts identified by high performance liquid chromatography analysis.

SNaB: spray-dried sodium caseinate + blackcurrant concentrate; FNaB: freeze-dried sodium caseinate + blackcurrant concentrate

8.5.2 Particle sizes distribution and bulk density

Figure 8.3 exhibits monomodal size distributions of the encapsulates. Table 8.1 shows the D[4,3] and the span values of the particles. The D[4,3] of SNaC (27.10 ± 0.74 µm) and SNaB (51.66 ± 0.53 µm) were significantly ($p < 0.001$) reduced by spray-drying comparing with original sodium caseinate (111.40 ± 2.70 µm). By contrast, the D[4,3] of FNaC (427.60 ± 3.85 µm) and FNaB (422.20 ± 2.28 µm)were significantly ($p < 0.001$) increased. Regarding the span values, both spray-dried and freeze-dried particles presented lower span values compared to the original sodium caseinate (3.07 ± 0.06) ($p < 0.05$). Moreover, spray-dried particles exhibited higher span values than freeze-dried particles ($p < 0.05$), while freeze-dried particles had higher D[4,3] than spray-dried particles. These results suggested that a wider range of particle size distribution and less homogeneity. Figure 8.3 presents the particle size characterisation of samples. The particle size of a material and its distribution are essential factors that can influence on the bulk density, water holding capacity, oil holding capacity on particles, and their further incorporation of protein ingredients (Tonon, Grosso, & Hubinger, 2011).

Spray-dried ingredients are regular in terms of size and shape. The size is mainly attributed to the physical conditions of spray-drying, including atomiser, compressed air pressure and inlet temperature. By comparison, freeze-dried particles are irregular in their size and shape, which might be owing to the feed solution concentration, the fragileness of the formed flakey material, and the degree of grinding. The irregular size and shape of freeze-dried ingredients may generate more external voids that can result in higher bulk volume, which, in turn, leads to lower bulk density. This is consistent with the results observed in Chapter 7, showing that the bulk density of FNaB was lower than SNaB ($p < 0.05$). The higher bulk density of SNaB could be due to the hygroscopicity of spray-dried powder, which can lead to the formation of a lump of powder. Bulk density is an important physical property, which can affect food transport,

handling, and storage. The bulk density value depends on the size, shape, and surface characteristics of particles. Figure 8.4 shows the bulk density characteristics of particles. Comparing with the original sodium caseinate, both spray-dried and freeze-dried particles demonstrated significant lower ($p < 0.001$) bulk density values. Moreover, SNaB showed

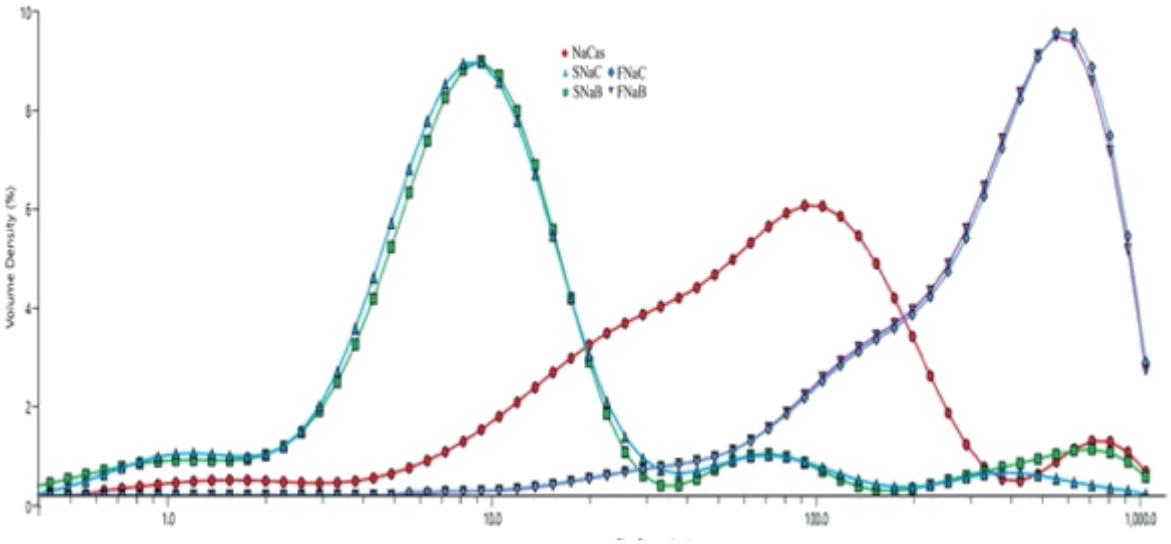

Figure 8.3 Particle sizes distribution by dynamic lighter scattering.

SNaB: spray-dried sodium caseinate + blackcurrant concentrate; SNaC: spray-dried sodium caseinate + imitation blackcurrant juice; FNaB: freeze-dried sodium caseinate + blackcurrant concentrate; FNaC: freeze-dried sodium caseinate+ imitation blackcurrant juice

significant higher bulk density values ($p < 0.001$) than FNaB.

Table 8.1 Particle size distribution

	$D_{[4,3]}$ (µm)	Span
NaCas	111.40 ± 2.70^{b}	3.07 ± 0.06^{B}
SNaC	27.1 ± 0.74^{d}	2.93 ± 0.04^{A}
SNaB	51.66 ± 0.53^{c}	2.87 ± 0.08^{A}
FNaC	427.60 ± 3.85^{a}	1.76 ± 0.01^{C}
FNaB	422.20 ± 2.28^{a}	1.54 ± 0.08^{D}

Values are expressed as means ± standard deviation (n = 3). Values in the same column with different letters differ significantly ($p < 0.05$). SNaB: spray-dried sodium caseinate + blackcurrant concentrate; SNaC: spray-dried sodium caseinate + imitation blackcurrant juice; FNaB: freeze-dried sodium caseinate + blackcurrant concentrate; FNaC: freeze-dried sodium caseinate+ imitation blackcurrant juice.

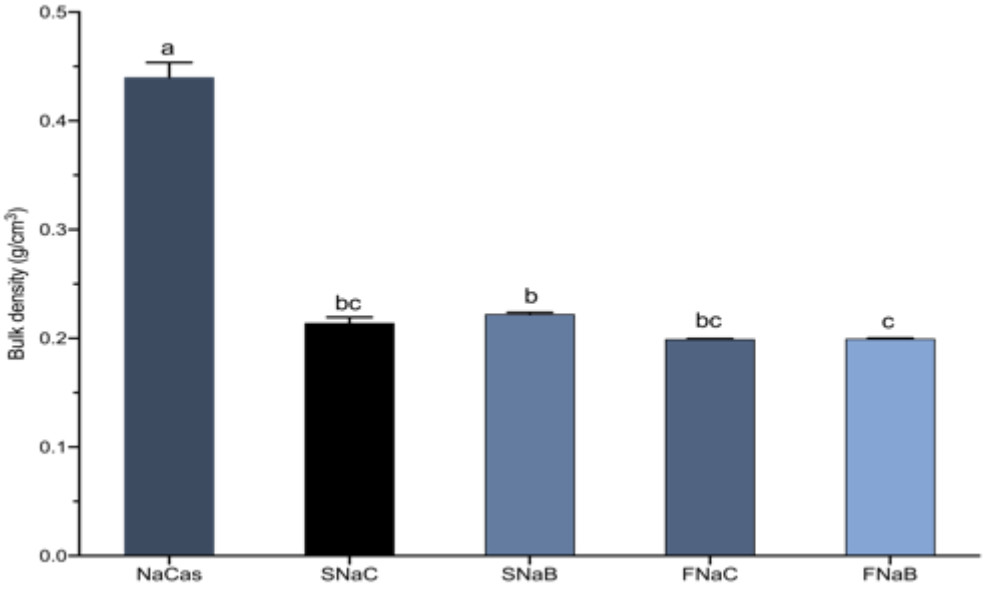

Figure 8.4 Bulk density (g/cm^3) of the novel functional ingredients.

Values = Means ± standard deviations (n = 3). Bars with the same letter do not differ significantly (p > 0.05). NaCas: sodium caseinate; SNaB: spray-dried sodium caseinate + blackcurrant concentrate; SNaC: spray-dried sodium caseinate + imitation blackcurrant juice; FNaB: freeze-dried sodium caseinate + blackcurrant concentrate; FNaC: freeze-dried sodium caseinate+ imitation blackcurrant juice.

8.5.3 Morphology, water holding capacity, and oil holding capacity

Figure 8.5 exhibits the surface morphology of the particle samples. It was obvious that drying strategies were the main factors affecting the shape and size of the particles. Through the spray-drying strategy, the samples obtained flour-like and moisture absorbable powders, due mainly to the huge surface areas. There was no obvious difference in the shape or size of the spray-dried particles. While through freeze-drying strategy the samples obtained flakey powders, which can be attributed to the difference in the drying processes themselves. However, freeze-drying produced lager flakey particles (10 µm vs. 100 µm). Meanwhile, FNaB exhibited more fragileness by easily crushed into small and tiny particles. A possible explanation for this is due to the addition of blackcurrant concentrate, leading to the formation of a fragile structure.

Figure 8.6 shows the water holding capacity and oil holding capacity of the ingredients. The water holding capacity of SNaC and SNaB ingredients were significantly ($p < 0.001$) higher compared to the original sodium caseinate and FNaC, and FNaB. Moreover, the water holding capacity of SNaB was lower than SNaC ($p < 0.05$). The water holding capacity measurement is based on the amount of physical sedimentation under the effect of centrifugal forces, which is a direct reflection of conjugates and/or complexes formation (Wasswa *et al.*, 2007). Under the spray-drying condition, the results indicated that more conjugates and complexes formed compared to the control. While under freeze-drying condition, the particles retained water solubility at the similar lever with control. Only under spray-drying condition, the addition of blackcurrant concentrate decreased the water holding capacity of SNaB, which can be attribute the competing of binding sites between proteins molecules and blackcurrant anthocyanins. Freeze-dried samples formed more voids between particles, which is in

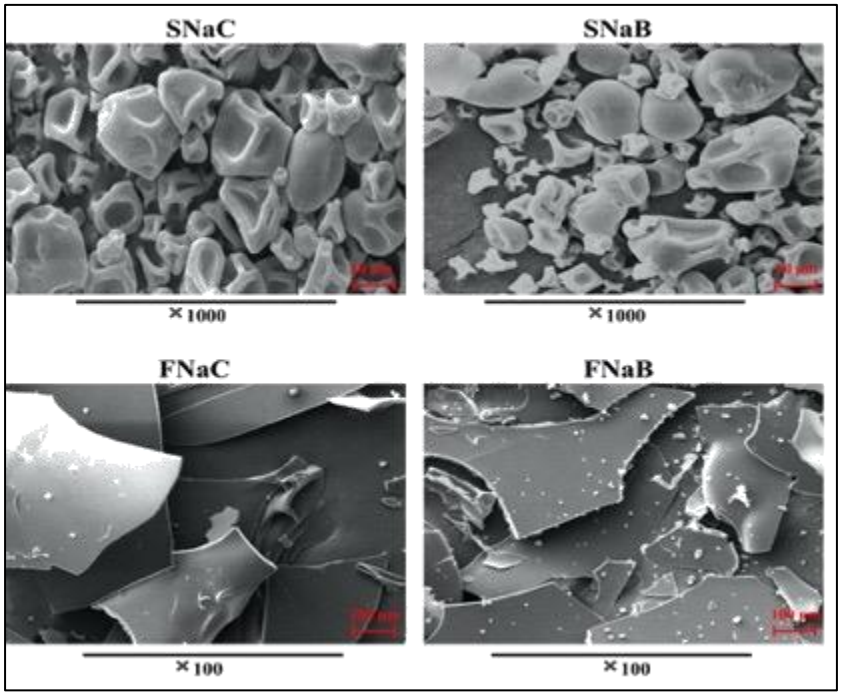

Figure 8.5 Morphology of spray-dried and freeze-dried powders at x 1,000 or x 100 magnification.

SNaB: spray-dried sodium caseinate + blackcurrant concentrate; SNaC: spray-dried sodium caseinate + imitation blackcurrant juice; FNaB: freeze-dried sodium caseinate + blackcurrant concentrate; FNaC: freeze-dried sodium caseinate+ imitation blackcurrant juice.

agreement with the higher oil holding capacity of freeze-dried FNaC and FNaB compared to spray-dried samples (SNaC and SNaB) ($p < 0.001$).

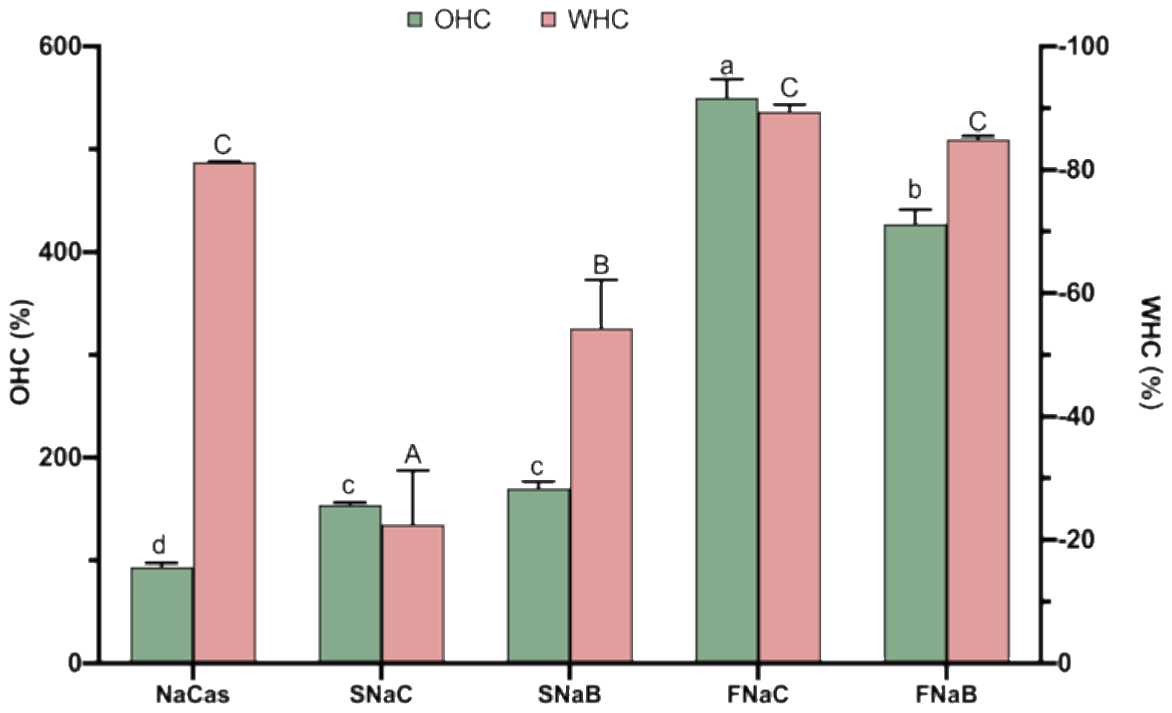

Figure 8.6 The oil holding capacity and water holding capacity of functional ingredients

OHC: oil holding capacity; WHC: water holding capacity; SNaB: spray-dried sodium caseinate + blackcurrant concentrate; SNaC: spray-dried sodium caseinate + imitation blackcurrant juice; FNaB: freeze-dried sodium caseinate + blackcurrant concentrate; FNaC: freeze-dried sodium caseinate+ imitation blackcurrant juice. Values = Means ± standard deviations (n = 3). Bars with different letters differ significantly (p < 0.05).

8.5.4 Changes in functional properties

Foam capacity and stability are important parameters for protein ingredient, which can reveal the potential of the protein ingredient to affect the quality and texture of products (Deotale, Dutta, Moses, Balasubramaniam, & Anandharamakrishnan, 2020). The morphological variations between the different foams assist in clarifying the differences in foaming capability and stability between samples (Lonchamp, Clegg, & Euston, 2019). As shown in Figure 8.7, the microscopic profiles provide the physical elucidation of the effects of blackcurrant anthocyanins on the foaming potential of sodium caseinate. The four formulated protein ingredients (SNaC, SNaB, FNaC, and FNaB) formed distinctly larger air bubbles, and more

uniform bubble sizes comparing with the sodium caseinate-based foam ($p < 0.05$). Freeze-dried samples (FNaC and FNaB) formed larger bubbles than spray-dried samples ($p < 0.05$), which is consistent with the observation that freeze-dried samples had a higher foamability than spray-dried samples. After standing for 30 min, the foam in FNaB had more air bubbles than FNaC, as the same case of SNaB was ($p < 0.05$). Moreover, SNaB had comparatively more bubbles than FNaB ($p < 0.05$). The foaming capacity of a protein is based on the rapid diffusion of the partially unfolded protein at the air-water interface, which decreases the surface tension of the air bubbles (Wang, Lin, & Yang, 2019). Herein, once sodium caseinate is mixed with blackcurrant concentrate, the blackcurrant anthocyanins in an aqueous solution begin to compete the binding sites on macromolecules, affecting the interaction among macromolecules. As a result, there was a formation of different complexes and/or conjugates. Through spray-drying and freeze-drying conditions, the complexes and/or conjugates were turned into the form of particles with different shapes and sizes with different rehydration properties (Emami, Vatanara, Park, & Na, 2018). Therefore, when the dried protein ingredients are reconstituted, the newly exposed binding sites on the macromolecules combine with the free water-soluble anthocyanin molecules. Hence, functional properties of sodium caseinate, such as foaming capacity and stability, have been altered.

The foaming capacity (0 min) and foam stability (30 min) of the protein ingredients have been shown in Figure 8.8. Spray- and freeze-drying techniques significantly increased the foaming ability of samples, in particular of freeze-dried samples, with the highest foaming ability ($p < 0.05$) among all ingredients. The most probable explanation for this finding is that the drying conditions partially unfold the protein molecules, and then the addition of blackcurrant anthocyanins further unfolds the structures of sodium caseinate thoroughly, ultimately enhancing the foaming capacity of the protein particles (Bordenave, Hamaker, & Ferruzzi, 2014). The foaming stability of SNaB and FNaB was considerably greater than the

corresponding control groups ($p < 0.05$). This can be attributed to the denaturation of natural protein molecules, and the further interactions of protein molecules with anthocyanins via covalent or non-covalent interactions. A complex formation between denatured protein and anthocyanin molecules can lead to a thicker protein absorption on a film, benefiting the foam stability (Ramos, Fernandes, Silva, Pintado, & Malcata, 2012). In addition, the foam stability of SNaB was significantly higher than FNaB ($p < 0.05$). This can be due to the more surface anthocyanins involved in the foam stability existing on the surface of spray-dried particles.

8.6 Conclusion

In this study, sodium caseinate was functionalised by inclusion of blackcurrant concentrate via spray-drying or freeze-drying technique to develop a novel protein ingredient. The changes in functional properties of this protein ingredient were analysed. It was found that both spray-drying and freeze-drying effectively encapsulated the anthocyanins from blackcurrant concentrate, when using sodium caseinate as a carrier. Different encapsulation strategies were attributed to the difference in the physical and functional characteristics of these encapsulates. A deep insight into their unique physical and functional characteristics of these ingredients is crucial for their further applications in appropriate food matrix. Both spray-dried particles and freeze-dried particles have their unique physical properties, including bulk density, water holding capacity, oil holding capacity, particles shapes and sizes, foamability and foam stability. For the future work, it would be interesting to consider the incorporation of these novel functional protein ingredients into real food products, such as cookies and muffins. In Chapter 9, these novel protein ingredients were further incorporated into cookies to determine the effects of the functional ingredients on cookie products.

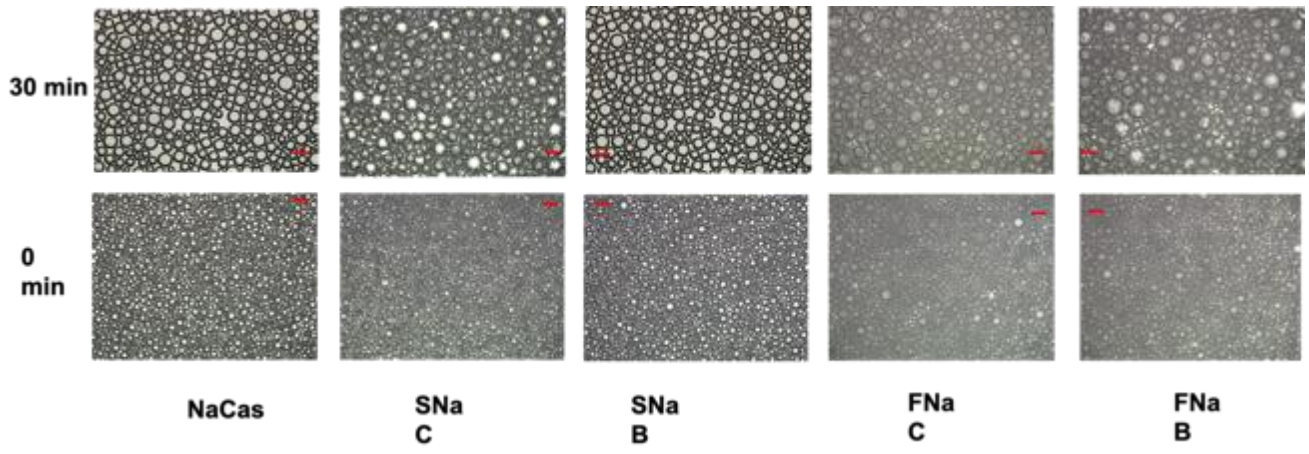

Figure 8.8 Microscope structure of foam (0 min and 30 min).

SNaB: spray-dried sodium caseinate + blackcurrant concentrate; SNaC: spray-dried sodium caseinate + imitation blackcurrant juice; FNaB: freeze-dried sodium caseinate + blackcurrant concentrate; FNaC: freeze-dried sodium caseinate+ imitation blackcurrant juice.

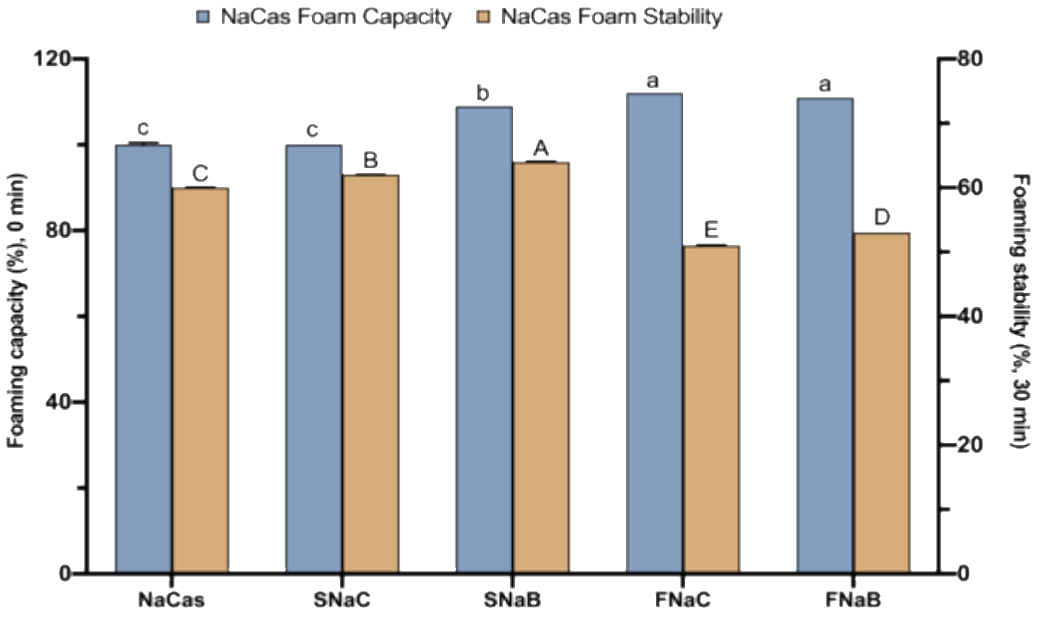

Figure 8.7 Foamability and foam stability.

SNaB: spray-dried sodium caseinate + blackcurrant concentrate; SNaC: spray-dried sodium caseinate + imitation blackcurrant juice; FNaB: freeze-dried sodium caseinate + blackcurrant concentrate; FNaC: freeze-dried sodium caseinate+ imitation blackcurrant juice.

Chapter 9

Sodium caseinate-blackcurrant concentrate powder obtained by spray-drying or freeze-drying for delivering structural and health benefits of cookies

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9.1 Abstract

The aqueous combination of sodium caseinate and blackcurrant concentrate has been turned into a powder form via spray-drying (SNaB) or freeze-drying (FNaB). The obtained powders differing in physical and functional properties have been found to have the potential to be used as a functional additive for the purposes of protein nutrition, antioxidant, colourant, reducing carbohydrate content. In this chapter, the obtained powders (SNaB and FNaB) were incorporated into a cookie recipe at a replacement level of 0%, 5%, 10%, and 15%. The effects of SNaB and FNaB powders on the physical and nutritional properties of the cookie products were evaluated. The colour, and texture properties of the cookies, important factors for customer acceptance, have been changed with the addition of these protein ingredients. The cookies' protein nutrition was also improved with the increase in protein content, and the antioxidant intake increased with the addition of the novel protein ingredients. The addition of the protein ingredients led to hypoglycaemic action. Interestingly, SNaB and FNaB formulated into a cookie recipe have differential effects on the properties of the cookies.

9.2 Graphic abstract

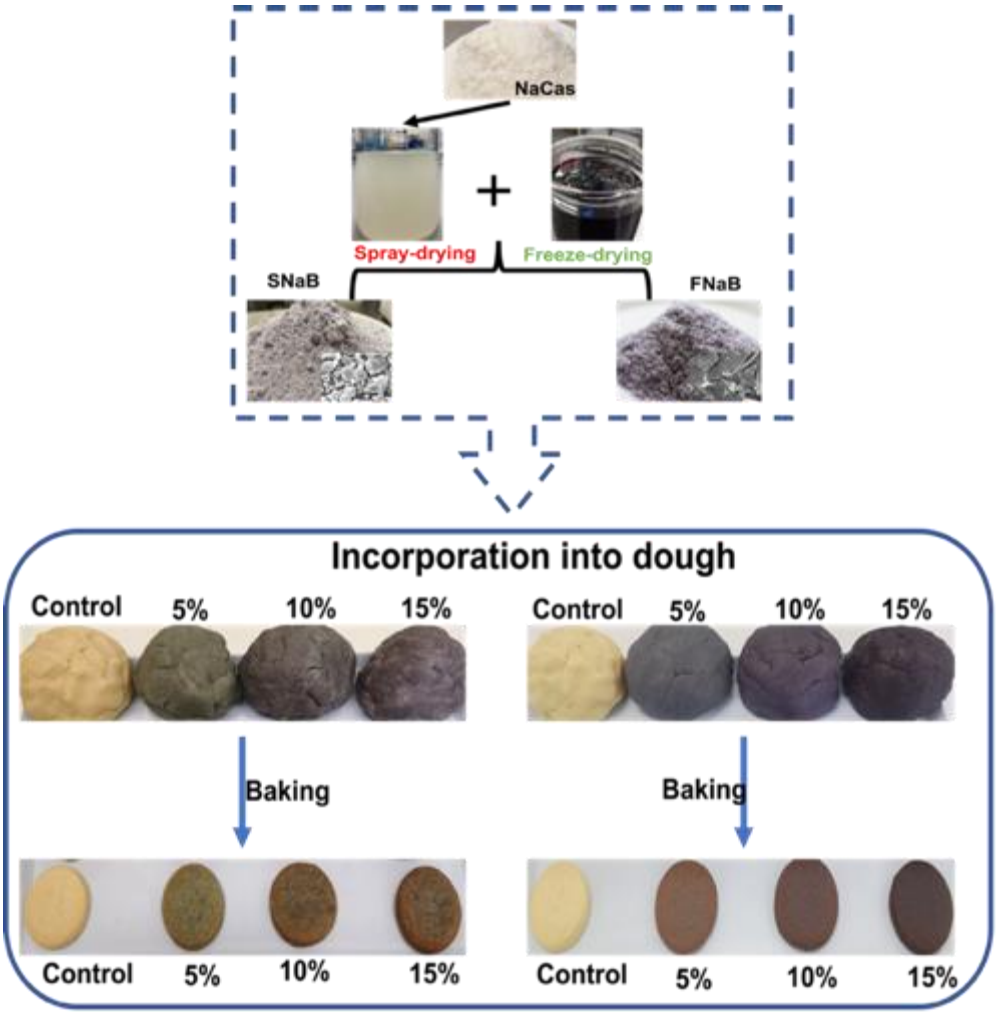

Figure 9.1 Overview of sodium casennate-blackcurrant concentrate based functional cookies

9.3 Introduction

Global societies are negatively impacted by the increasing chronic diseases such as cardiovascular disease, cancers, diabetes, and obesity, in all age groups, and this has been described as a silent global pandemic (Meetoo, 2008). A low carbohydrates and high protein diet with the addition of fruit polyphenols has become a pattern in recent years as a possible strategy to avoid the incidence of these chronic diseases (Hemler & Hu, 2019; Mahato, Sharma, Sinha, & Cho, 2018; Pallazola *et al.*, 2019). Commercially, blackcurrants are grown mainly for processing, especially for juice production, and the obtained blackcurrant juice concentrate is a rich source of polyphenols (Laaksonen *et al.*, 2014). Blackcurrant polyphenols

have been proved possessing various specific human health benefits, such as antioxidant activity, anti-inflammation activity, neuroprotective actions, anti-obesity, and anti-cancer properties (Cortez & Gonzalez de Mejia, 2019; Karjalainen *et al.*, 2009). Sodium caseinate is a commonly available milk protein ingredient, adding texture to food products while improving the protein content of food products (bakery, yogurt, ice cream, confectionery) (Chandan, 2011). Novel protein ingredients have been developed in a food-compatible manner by combining sodium caseinate with blackcurrant concentrate (Figure 9.1). Spray-drying and freeze-drying, as common encapsulation strategies (Hu *et al.*, 2018; Pudziuvelyte *et al.*, 2020), were used to turn the combined liquid solutions into two different novel protein powder ingredients, SNaB and FNaB, respectively.

Numerous studies have investigated the encapsulation of bioactive compounds in whey or soy protein, addressing the techniques and effects of operational parameters on the prevention of bioactive compounds from degradation (Tumbas Šaponjac *et al.*, 2016). However, no study, if any, has dealt with the encapsulation of bioactive compounds in sodium caseinate, and the further incorporation of them into real food systems. Previously, these combinations have been shown to enhance the stability, antioxidant activity, and the *in vitro* release of polyphenols by delivering them to lower parts of the gastrointestinal tract, and increasing their bioavailability and bioaccessibility (Huang *et al.*, 2019). The fact that the functional components, such as anthocyanins, encapsulated inside these ingredients (SNaB, FNaB) are still water-soluble in an aqueous solution, and this is remarkable when considering the further application of these functional ingredients (Mäkilä *et al.*, 2016). Thus, the choice of an appropriate food matrix is of vital importance. A solid food matrix, such as dough for cookie or bread, is one option. In this case, snack foods that provide macronutrients (protein and carbohydrate) with the extra health benefits of blackcurrant have been developed, providing a means of improving the quality of diet.

This chapter was designed to investigate the possibility of developing a functional cookie supplemented with sodium caseinate-encapsulated blackcurrant polyphenols. To understand the effects of different powders on dough and cookie properties, different wheat flour replacement formulations (0%, 5%, 10%, and 15%) were prepared, and the physicochemical properties (colour, texture, and proximate analysis) and nutritional characterics (total phenolic content, antioxidant activity, and the predicted *in vitro* glycaemic response) of the cookies were examined.

9.4 Methods

9.4.1 Cookie production

Cookie samples were prepared according to the protocol in 3.2.

9.4.2 Textural properties of dough and cookies

Texture measurement was carried out by following the methods described in 3.20.

9.4.3 Colour profiles of dough and cookies

Colour measurement were conducted as the method described in 3.6.

9.4.4 Ash content

The ash content was measured as described in 3.4.

9.4.5 Total carbon and protein content

The ash content was measured as described in 3.5.

9.4.6 Sample extraction

The chemical extraction was performed as described in 3.7.

9.4.7 Simulation of the *in vitro* digestion process and glycaemic glucose equivalent

The simulation of the *in vitro* digestion process and glycaemic glucose equivalent assay was carried out as described in 3.8.

9.4.8 Determination of total phenolic content

The total phenolic content was determined as described in 3.10.

9.4.9 Antioxidant activity

The antioxidant activity measurement was carried out as described in 3.12.

9.4.10 Statistical analysis

Statistical analysis was performed as described in 3.24.

9.5 Results and discussion

9.5.1 Physical characteristics and proximal compositions of cookies

The texture properties of dough are fundamental characteristics for cookie formulation as well as cookie quality. It is worthy to note that if the dough is very soft or firm, it is hard to manipulate (Mamat & Hill, 2014). Table 9.1 displays the texture characteristics of the formulated dough and cookie samples. At increasing levels of SNaB and FNaB, the textural characteristics of the samples significantly changed ($p < 0.05$). Before baking, as the amount of SNaB and FNaB encapsulates increased, the hardness of the dough increased significantly. The hardness of FNaB-enriched cookie dough was higher than the corresponding SNaB-enriched cookie dough ($p < 0.05$), and 15% FNaB was the hardest dough sample with a value of 3669.70 g. These results are in agreement with the conclusions from Chapter 8, which showed that both SNaB and FNaB had a greater water holding capacity and oil holding capacity. The moisture content of both SNaB- and FNaB-enriched cookies was higher than the

moisture content of the control cookies ($p < 0.05$). The water holding capacity is responsible for the higher moisture content of the cookies.

SNaB-enriched cookies had larger diameters than the corresponding FNaB-enriched cookies and the control group ($p < 0.05$). Moreover, as the percentage of SNaB incorporated into cookies increased, the resulting cookies had increasingly bigger diameters, while the opposite was true for the FNaB-enriched cookies. The thickness parameters of 5% SNaB- and FNaB-enriched cookies were higher than that of the control cookie, while 10% and 15% FNaB- and SNaB-enriched cookies were lower than control cookie ($p < 0.05$). The spread factor of a cookie is affected by the viscosity of dough and the water holding capacity of the encapsulates incorporated into the cookie dough allowing it to expand in volume (Adeola & Ohizua, 2018; Barak, Mudgil, & Khatkar, 2014; Belorio, Sahagún, & Gómez, 2019). van der Sman and Renzetti (2019) has reported that ingredients with a lower hydration holding capacity produced cookies with larger diameters since the excess water in the dough mixture was available to dissolve the sugar, reduced the initial viscosity of the dough and allowed more expansion during the baking process. This appears to be the case in this study, observing that the encapsulates of SNaB with a lower water holding capacity produced larger diameter cookies than the encapsulates of FNaB.

Protein content and the ratio of C/N of the samples is outlined in Table 9.2. Incorporation of the encapsulated SNaB and FNaB powders in cookies increased their protein content and decreased the ratio of C/N in comparison to the control sample. 15% SNaB- and 15% FNaB-enriched cookies had the highest protein content as well as the lowest C/N ratio among all treatments ($p < 0.05$). In addition, a positive correlation between the C/N ratio and diameter of cookies ($R^2 = 0.694$, $p < 0.05$) was obtained, which indicated that the interaction between the protein and starch might affect the water holding capacity of the encapsulates in the

dough, thus resulting in a difference in the textural properties of the resulting cookies. This is in agreement with the finding of Pourmohammadi *et al.* (2019).

9.5.2 Colour profiles

Table 9.3 shows the *L*, a*, b*,* and *ΔE* values for the dough, and the corresponding cookie surface, and ground cookies. The value of *L** indicates the lightness ranging from 100 (the lightest) to 0 (the darkest). The value of *a** represents the redness (positive value) and greenness (negative value), while the value of *b** reveals the yellowness (positive value) and blueness (negative value). The value of △E (colour difference) represents the colour distance within the groups (Khoo *et al.*, 2017). Cookies and dough formulated with SNaB and FNaB encapsulates were darker compared with the control group, having lower *L** values ($p < 0.05$). Higher *a** values (greater redness), and lower *b** values (less yellowness) were obtained in dough and cookies formulated with encapsulates in concentration-dependent manners, when compared to the control group ($p < 0.05$). In addition, cookies containing FNaB-enriched powder were lighter, more red and yellow when compared to the corresponding SNaB-enriched cookies ($p < 0.05$). Positive correlations between total phenolic content values and the ΔE values of doughs ($R^2 = 0.572$, $p < 0.05$), the baked cookies ($R^2 = 0.742$, $p < 0.05$), and the ground cookies ($R^2 = 0.727$, $p < 0.05$) were observed. This suggested that the colour parameters of the dough and cookies were mainly influenced by the amount of encapsulates, and hence, the amount of anthocyanins incorporated into the cookies. It is notable that the ΔE values of SNaB-enriched cookies were lower than the corresponding FNaB-enriched groups. These results were consistent with our previous study, which showed that compared with freeze-drying, spray-drying preserved the surface anthocyanin content of sodium caseinate blackcurrant concentrate encapsulates more effectively (Wu, Hui, Mu*, et al.*, 2021). Surface anthocyanins content already known to be another factor leading to the difference in the colour parameters of cookies (Mahloko, Silungwe, Mashau, & Kgatla, 2019).

Table 9.1 Physical characteristics of dough and cookies

	Dough		Cookies		
	Dough hardness (g)	Baking loss (%)	Cookie hardness (g)	Diameter (%)	Thickness (%)
Control	756.74 ± 24.68^{f}	8.69 ± 0.18^{ab}	16138.35 ± 1026.59^{ab}	13.00 ± 1.35^{c}	59.80 ± 3.31^{c}
5% SNaB	1491.52 ± 35.13^{e}	9.01 ± 0.33^{a}	17188.50 ± 936.97^{ab}	19.40 ± 1.79^{b}	80.40 ± 7.47^{a}
10% SNaB	2312.35 ± 59.86^{c}	9.07 ± 0.23^{a}	17656.30 ± 1500.18^{a}	22.70 ± 0.49^{a}	40.50 ± 7.19^{ab}
15% SNaB	1812.74 ± 34.37^{d}	6.29 ± 2.23^{b}	15728.96 ± 955.24^{ab}	22.10 ± 1.55^{a}	50.20 ± 5.65^{b}
5% FNaB	1525.88 ± 61.69^{e}	7.21 ± 0.33^{ab}	11703.82 ± 1388.15^{b}	12.30 ± 0.57^{c}	108.00 ± 2.35^{c}
10% FNaB	2648.79 ± 47.32^{b}	9.14 ± 0.31^{a}	14118.03 ± 1599.28^{ab}	13.60 ± 0.72^{c}	59.80 ± 2.91^{b}
15% FNaB	3669.70 ± 148.62^{a}	8.85 ± 2.27^{a}	13885.89 ± 1308.21^{b}	8.73 ± 1.02^{d}	73.80 ± 11.30^{ab}

Mean ± standard deviation. Values within a vertical column followed by the different letter are significantly different from each other ($p < 0.05$). SNaB: spray-drying + sodium caseinate + blackcurrant concentrate; FNaB: freeze-drying + sodium caseinate + blackcurrant concentrate.

Table 9.2 Proximal analysis of cookies

	Ash (%)	Moisture (%)	Protein (%)	C/N
Control	1.31 ± 0.07^{b}	8.36 ± 0.07^{d}	7.60 ± 0.24^{d}	43.97 ± 1.48^{a}
5% SNaB	1.17 ± 0.10^{b}	11.36 ± 0.15^{a}	9.91 ± 0.26^{c}	35.28 ± 0.93^{b}
10% SNaB	1.32 ± 0.05^{ab}	10.24 ± 0.04^{b}	11.71 ± 0.03^{a}	29.54 ± 0.07^{c}
15% SNaB	1.30 ± 0.23^{ab}	9.10 ± 0.04^{c}	13.43 ± 0.12^{b}	25.24 ± 0.32^{d}
5% FNaB	1.20 ± 0.04^{b}	9.13 ± 0.12^{c}	9.74 ± 0.03^{c}	34.49 ± 0.12^{b}
10% FNaB	1.33 ± 0.04^{ab}	9.89 ± 0.18^{bc}	11.67 ± 0.04^{b}	29.23 ± 0.14^{c}
15% FNaB	1.43± 0.04^{a}	9.72 ± 0.15^{bc}	13.51 ± 0.04^{a}	25.27 ± 0.08^{d}

Means ± standard deviations (n = 3). Values in the same column with different letters differ significantly (p < 0.05). SNaB: spray-dried sodium caseinate + blackcurrant concentrate; FNaB: freeze-dried sodium caseinate + blackcurrant concentrate

Table 9.3 Colour profile (dough, cookies, cookie powder)

		*L**	*a**	*b**	*ΔE*
Control	Dough	66.04 ± 0.78^{a}	0.05 ± 0.18^{c}	15.01 ± 0.76^{a}	25.54 ± 0.81^{a}
	Cookie	76.14 ± 0.06^{a}	-1.77 ± 0.08^{c}	25.54 ± 0.71^{a}	39.97 ± 0.49^{a}
	Cookie powder	79.98 ± 0.52^{a**}	-1.62 ± 0.06^{f}	24.01 ± 0.32^{a}	42.04 ± 0.25^{a**}
5% SNaB	Dough	54.31 ± 0.39^{b}	-0.46 ± 0.06^{c}	0.58 ± 0.07^{c}	11.26 ± 0.32^{b}
	Cookie	59.11 ± 1.12^{c**}	-0.85 ± 0.37^{bc}	12.45 ± 0.96^{bc**}	19.12 ± 0.95^{c**}
	Cookie powder	66.42 ± 2.48^{cd**}	-1.61 ± 0.17^{f}	14.19 ± 1.13^{bc}	25.92 ± 2.58^{bc*}
10% SNaB	Dough	52.43 ± 0.53^{b}	1.22 ± 0.04^{b}	-1.17 ± 0.15^{d}	8.96 ± 0.36^{cd}
	Cookie	53.05 ± 0.32^{d}	2.60 ± 0.23^{a**}	10.80 ± 0.56^{c**}	13.04 ± 0.20^{d**}
	Cookie powder	62.54 ± 0.41^{d**}	0.49 ± 0.06^{d**}	12.50 ± 0.06c**	21.31 ± 0.35^{c**}
15% SNaB	Dough	47.93 ± 0.72^{c}	1.27 ± 0.21^{ab}	-3.69 ± 0.05^{f}	7.69 ± 0.30^{c}
	Cookie	48.12 ± 0.79^{e}	3.07 ± 0.33^{a**}	6.77 ± 1.37^{d**}	7.58 ± 1.12^{e}
	Cookie powder	58.69 ± 0.46^{c**}	1.78 ± 0.10^{b**}	10.88 ± 0.34^{d**}	17.04 ± 0.53^{cd**}
5% FNaB	Dough	53.34 ± 0.12^{b}	0.00 ± 0.02^{cd}	1.50 ± 0.03^{b}	10.26 ± 0.10^{b}
	Cookie	66.00 ± 0.50^{b**}	0.77 ± 0.18^{b**}	16.24 ± 0.23^{b**}	26.03 ± 0.29^{b**}
	Cookie powder	70.42 ± 0.49^{b**}	-0.47 ± 0.14^{e**}	15.59 ± 0.29^{b}	29.51 ± 0.31^{b**}
10% FNaB	Dough	53.50 ± 0.05^{b}	2.24 ± 0.01^{a}	-0.58 ± 0.06^{d}	9.06 ± 0.04^{cd}
	Cookie	53.35 ± 1.33^{cd}	3.45 ± 1.21^{a}	9.90 ± 2.41^{cd**}	12.37 ± 2.08^{de}
	Cookie powder	63.97 ± 0.34^{d**}	1.15 ± 0.11^{c}	12.73 ± 0.21c	22.36 ± 0.27^{c**}
15% FNaB	Dough	52.86 ± 0.11b	2.11 ± 0.04^{a}	-1.99 ± 0.02^{e}	8.95 ± 0.10^{cd}
	Cookie	47.90 ± 0.64^{e**}	4.46 ± 0.25^{a**}	7.66 ± 0.38^{d**}	7.44 ± 0.37^{e**}
	Cookie powder	61.22 ± 0.55^{d**}	3.18 ± 0.15^{a**}	12.49 ± 0.31^{c**}	19.56 ± 0.55^{cd**}

Values in the same column with different letters differ significantly ($p < 0.05$). * $p < 0.05$; ** $p < 0.01$. SNaB: spray-dried sodium caseinate + blackcurrant concentrate; FNaB: freeze-dried sodium caseinate + blackcurrant concentrate

9.5.3 Total phenolic content content and antioxidant capacity of cookies

From the data presented in Figure 9.2, the total phenolic content values were significantly ($p < 0.05$) different and increased in a linear manner as an increasing amount of encapsulates were incorporated into cookies. Cookies with encapsulates were enriched in polyphenols, ranging from 36.98 µg gallic aid equivalent/g to 86.12 µg gallic aid equivalent/g, with the cookies enriched with 15% SNaB (86.12 µg gallic aid equivalent/g) and 15% FNaB (77.30 µg gallic aid equivalent/g), having 3- and 2.5-fold higher total phenolic content values compared with the control cookies, respectively ($p < 0.05$). After undergoing the *in vitro* digestion, the total phenolic content in the intestinal digesta of cookies enriched with encapsulates had increased dramatically ($p < 0.05$), ranging from 464.11 to 622.88 µg gallic aid equivalent/g, which was significantly higher than the control group ($p < 0.05$). The total phenolic content of the intestinal digesta of 15% SNaB-enriched cookies was also higher than the corresponding 15% FNaB cookies, having a 2- and 1.5-fold higher total phenolic content than the control group, respectively ($p < 0.05$). These observations support our conclusions in Chapter 5, showing that spray-drying prevented the encapsulated bioactive compounds from being degraded more effectively than freeze-drying. This confirms that sodium caseinate might be a good carrier for delivering phenolic compounds, which is also in support of previous studies, revealing that sodium caseinate exhibited a high release profile in intestinal fluids despite the encapsulates were prepared by different strategies (Chawda, Shi, Xue, & Young Quek, 2017). Significant correlations between total phenolic content and the protein content of cookies ($R^2 = 0.887$, $p < 0.05$), as well as C/N ratio ($R^2 = 0.856$, $p < 0.05$), total phenolic content and the ash content of cookies ($R^2 = 0.760$, $p < 0.05$) were observed (Table 9.4), indicating that apart from the phenolic compounds, the protein and mineral contents of the samples may contribute to the total phenolic content as well.

There is no single chemical assay that can accurately determine the contribution of the bioactive compounds to the total antioxidant activity of samples. Moreover, the antioxidant activity of bioactive compounds is highly influenced by the chemical transformations during the digestion (Gulcin, 2020; Guo *et al.*, 2017; Pudziuvelyte *et al.*, 2020). Herein, the DPPH radical scavenging ability and ferric reducing antioxidant power of the cookies were both employed to investigate the antioxidant activities in the samples. Figure 9.3 shows the antioxidant capacity of cookies as measured by the DPPH (Figure 9.3a) and FRAP assay (Figure 9.3b). The Pearson's correlation between antioxidant capacity and total phenolic content values were also observed. When the encapsulates of SNaB and FNaB were incorporated into cookies, their DPPH radical scavenging capacity and ferric reducing antioxidant power both increased significantly. Increasing the incorporated level of encapsulates into the cookies, from 5% to 15%, seemed to be no significant additional effect on the DPPH radical scavenging capacity, ranging between 5.40 and 5.50 µmoL TE/g, while this positively influenced the ferric reducing antioxidant power of the cookies. 15% FNaB-enriched cookies exhibited the highest reducing power (8.28 mmoL $FeSO_4$/g), followed by 15% SNaB-enriched cookies (7.16 mmoL $FeSO_4$/g), which was 2- and 2.5-fold higher than the control group, respectively ($p < 0.05$). This result is in line with the total phenolic content results. After *in vitro* digestion, the DPPH radical scavenging ability increased in all cookies, except for 15% FNaB-enriched cookies which decreased slightly compared with the corresponding undigested extract. Phenolic compounds responsible for ferric reduction may reduce or convert to certain metabolites with different chemical properties during digestion, as these bioactive compounds are sensitive to alkaline conditions (Altemimi, Lakhssassi, Baharlouei, Watson, & Lightfoot, 2017). However, apart from the 15% FNaB-enriched cookies, all cookies enriched with encapsulates increased their reducing power after the *in vitro* digestion. It is assumed that sodium caseinate protected blackcurrant concentrate from experiencing the oxidative stress under alkaline conditions.

The intestinal digesta of 15% SNaB-enriched cookies had the strongest scavenging ability (7.84 μmoL TE/g) as well as the highest FRAP values (7.84 mmoL $FeSO_4$/g) among all digested samples. These observations may be due to the more efficient technique of spray-drying. Significant correlations between total phenolic content and FRAP, and total phenolic content and DPPH values before and after the *in vitro* digestion ($p < 0.05$) were obtained, supporting hypothesis that the bioactive compounds are positively related to the increase in the antioxidant ability. In addition, the protein content, the C/N ratio, and the ash content were all make contributions to the antioxidant ability.

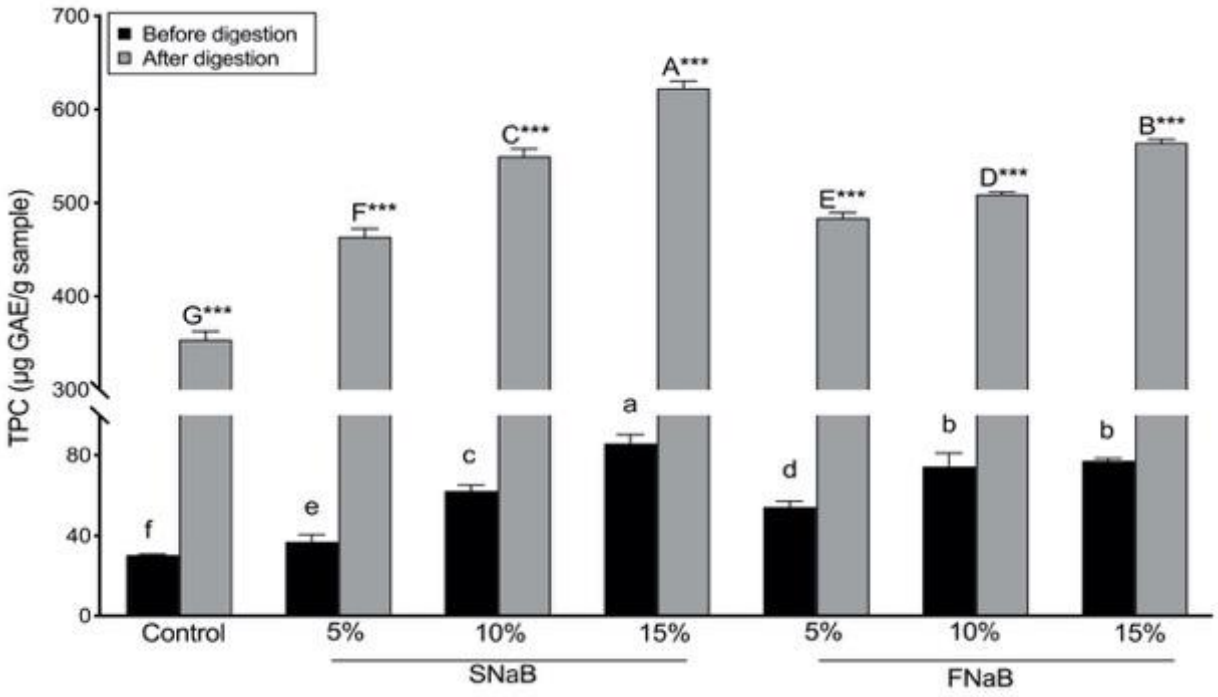

Figure 9.2 The total phenolic content (TPC) of cookies before and after the *in vitro* digestion. *Values before the digestion with different small letters, while values after the digestion with different up-letters are statistical different ($p < 0.05$). Comparison in a group is expressed by * $p < 0.05$ or ** $p < 0.01$. SNaB: spray-dried sodium caseinate + blackcurrant concentrate; FNaB: freeze-dried sodium caseinate + blackcurrant concentrate.*

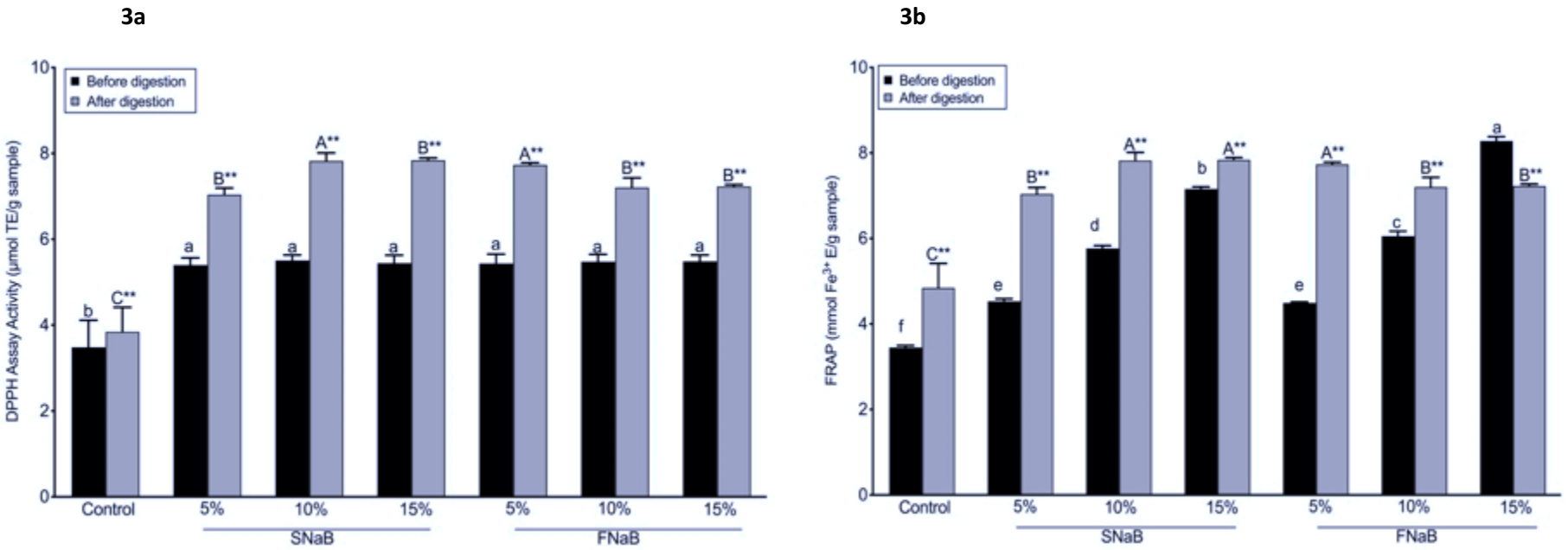

Figure 9.3 (3a) The DPPH and (3b) the FRAP values of cookies before and after the *in vitro* digestion.

Values before the digestion with different small letters, and values after the digestion with different uppercase letters are statistical different ($p < 0.05$). Comparison in a group is expressed by * $p < 0.05$ or ** $p < 0.01$. SNaB: spray-dried sodium caseinate + blackcurrant concentrate; FNaB: freeze-dried sodium caseinate + blackcurrant concentrate

Figure 9.4 Pearson's correlations between observed values

	TPC (before digestion)	TPC (after digestion)	DPPH (before digestion)	DPPH (after digestion)	FRAP (before digestion)	FRAP (after digestion)	AUC
TPC (after digestion)	0.907**						
DPPH (before digestion)	0.651	0.797*					
DPPH (after digestion)	0.673	0.838*	0.974**				
FRAP (before digestion)	0.896**	0.861*	0.607	0.571			
FRAP (after digestion)	0.679	0.846*	0.953**	0.996**	0.558		
AUC	-0.937**	-0.910**	-0.748	-0.721	-0.974**	-0.708	
Moisture content	-0.046	0.211	0.601	0.478	0.112	0.423	-0.169
Protein content	0.942**	0.955**	0.721	0.714	0.969**	0.708	-0.978**
C/N	-0.925**	-0.964**	-0.836*	-0.823*	-0.925**	-0.812*	0.973**
Ash content	0.872**	0.769*	0.568	0.532	0.936**	0.523	-0.925**
Hardness of cookie	-0.277	-0.056	-0.195	-0.197	-0.145	-0.195	0.258
Diameters of cookies	0.673	0.847*	0.977**	0.995**	0.576	0.989**	-0.719
Thickness of cookies	-0.288	-0.270	0.124	0.093	-0.269	0.070	0.159
Hardness of dough	0.068	0.347	0.238	0.336	-0.056	0.365	0.033
Baking loss	-0.362	-0.402	-0.128	-0.291	-0.138	-0.339	0.195
$\triangle E$ (dough)	-0.756*	-0.878**	-0.987**	-0.978**	-0.690	-0.963**	0.819*
$\triangle E$ (cookie)	-0.861*	-0.932**	-0.843*	-0.805*	-0.893**	-0.785*	0.932**
$\triangle E$ (cookie powder)	-0.853*	-0.957**	-0.883**	-0.868*	-0.848*	-0.855*	0.907**

$^{*}p < 0.05$, $^{**}p < 0.01$; AUC: area under curve; TPC: total phenolic content

Continuation of Table 9.4

	Moisture Content	Protein content	C/N	Ash content	Hardness of cookie	Diameters of cookies	Thickness of cookies	Hardness of dough	Baking loss	$\triangle E$ (dough)	$\triangle E$ (cookie)
Protein content	0.182										
C/N	-0.302	-0.982**									
Ash content	0.056	0.894**	-0.868*								
Hardness of cookie	0.396	-0.087	0.120	-0.205							
Diameters of cookies	0.527	0.726	-0.833*	0.526	-0.122						
Thickness of cookies	0.022	-0.313	0.234	-0.315	-0.708	0.031					
Hardness of dough	0.357	0.148	-0.180	-0.155	0.719	0.393	-0.548				
Baking loss	0.474	-0.241	0.205	0.048	0.341	-0.260	-0.180	-0.217			
$\triangle E$ (dough)	-0.508	-0.807	0.901**	-0.641	0.188	-0.983**	-0.026	-0.269	0.213		
$\triangle E$ (cookie)	-0.447	-0.956**	0.982**	-0.821*	-0.020	-0.831*	0.293	-0.260	0.092	0.897**	
$\triangle E$ (cookie powder)	-0.451	-0.939**	0.978**	-0.764*	-0.013	-0.892**	0.255	-0.341	0.185	0.935**	0.990**

$^{*}p < 0.05$, $^{**}p < 0.01$, TPC: total phenolic content

9.5.4 *In vitro* glycaemic response of cookies

Glycaemic glucose equivalent assay was conducted on the cookies to evaluate the reducing sugar released during a 120-min *in vitro* digestion. Figure 9.4a shows the glucose released calculated as the reducing sugar hydrolysed from starch by digestive enzymes. All cookie groups showed a sharp increase rate of reducing sugar released in the first 20 min of the digestion, and then during 20-120 min; the rate of reducing sugar released decreased or even being flattened out. Figure 9.4b exhibits the area under curve values of cookies. The lower area under curve values of cookies, the lower glycaemic response cookies had. Increasing the amount of NaB encapsulates in cookies showed a significant decrease of the released sugar, and thus decreased the *in vitro* glycaemic response, compared with the control cookies ($p < 0.05$). The lowest area under curve value was found in the 15% FNaB-enriched cookies (674 mg glucose/g) and 15% SNaB-enriched cookies (741 mg glucose/g), which were approximately 30% lower than the control groups ($p < 0.05$).

Negative correlations were observed between area under curve and protein content ($R^2 = 0.957$, $p < 0.05$). These results suggest that the bioactive compounds and protein in cookies contributed to the lower rate of reducing sugar released and the starch degradation of cookies. Phenolics are sensitive to the alkaline conditions during the digestion and produce reactive species, which can interact with the free amino groups. These reactions can change the physicochemical properties, such as the solubility, molecular weight, and the secondary or the tertiary structures of proteins in cookies (Lund & Ray, 2017).

Blackcurrants contain high amounts of fibres and phenolic compounds, which are the effective inhibitors of digestive enzymes (Jurgoński, Juśkiewicz, Zduńczyk, Matusevicius, & Kołodziejczyk, 2014). The inhibitory enzymes activities of these compounds can be due to the interactions between digestive enzymes and phenolics, such as the hydrogen bonding, hydrophobic interactions, and ionic interactions, forming the “inhibitor-enzyme” or the

"inhibitor-starch-enzyme" complex (Martinez-Gonzalez *et al.*, 2017). In addition, both SNaB- and FNaB-enriched cookies generated a lower glycaemic response, which further revealed that sodium caseinate was a good vehicle for delivering bioactive compounds with functional properties, including antioxidant and hypoglycaemic properties.

9.6 Conclusion

This chapter showed that incorporation of blackcurrant concentrate encapsulated in sodium caseinate into cookies improved the cookies' physical characteristics, including the texture and colour parameters of cookies, as well as functional properties, such as increased total phenolic content and antioxidant activity, and the predicted *in vitro* glycaemic response. Compared with freeze-drying, spray-drying gave better results in terms of the cookies' physical and functional properties. These results revealed that there is a good potential for the sodium caseinate-blackcurrant concentrate encapsulates to be utilised as a functional ingredient in food systems, owing to its high content of bioactive compounds, strong antioxidant capacity. Future research should investigate the consumer acceptance of using this to enrich products, and further determine its functional properties via *in vivo* studies.

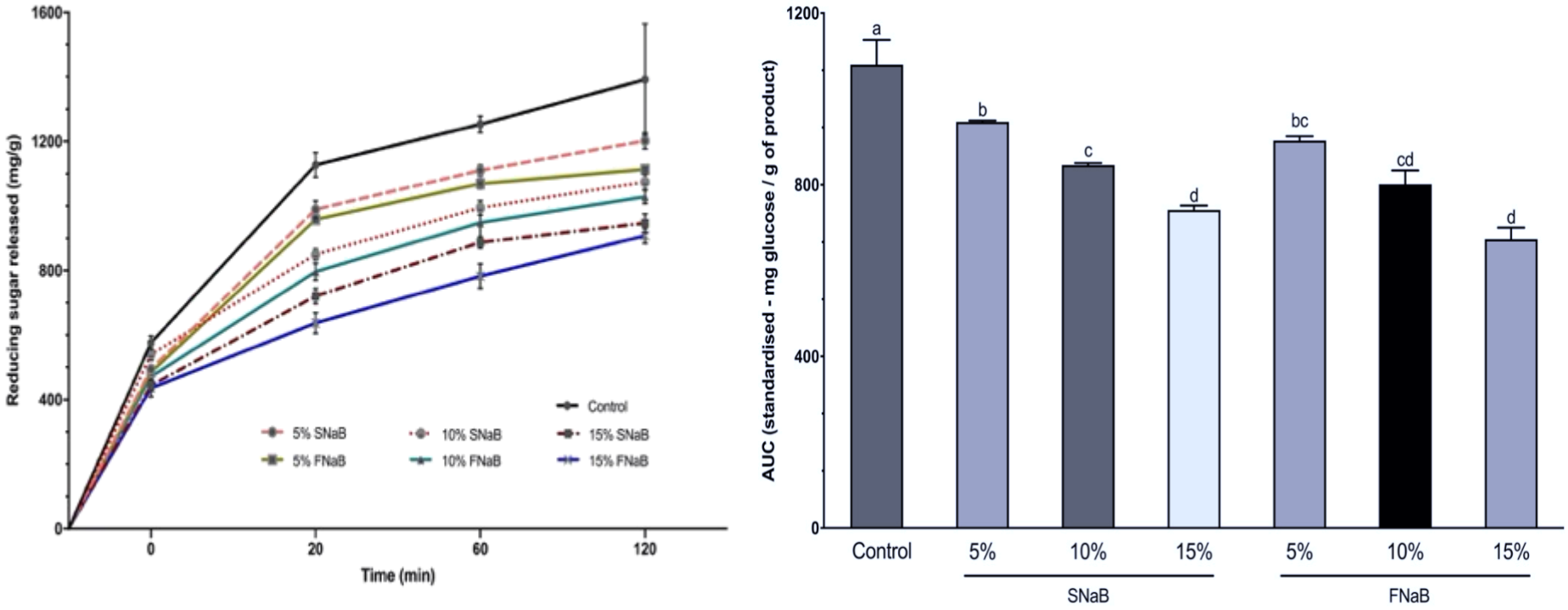

Figure 9.5 Reducing sugar content after simulated *in the vitro* digestion.

(a)The reducing sugar released of cookies during a 120-min in vitro digestion; (b) The area under curve (AUC) of cookies during in vitro digestion. Bars with different letters differ significantly ($p < 0.05$). SNaB: spray-dried sodium caseinate + blackcurrant concentrate; FNaB: freeze-dried sodium caseinate + blackcurrant concentrate

Chapter 10

General conclusions and future research

10.1 Longitudinal study

Chapters 4 and 5 focused on the longitudinal study of the physicochemical and nutritional properties of whey protein-based blackcurrant concentrate encapsulates. These encapsulates were further incorporated into cookies. As shown in Chapter 6, the physicochemical and nutritional properties of these encapsulate-enriched cookies was evaluated. From ingredients development to practical application approach, Chapters 4, 5, and 6 provided a holistic view of the development of the functional food.

Chapter 4 illustrated the protein content, colour profile, antioxidant activity, and encapsulation efficacy of the whey protein-based blackcurrant concentrate encapsulates obtained via spray- and freeze-drying. Collectively, α-amylase inhibition assay, molecular docking study, and glyceamic glucose equivalent assay suggested that the interactions of the main blackcurrant anthocyanins with α-amylase may be a potential explanation for reducing sugar released.

Chapter 5 defined both the functional (water holding capacity, oil holding capacity, foamability, foam stability, and bulk density), and anti-cancer properties of whey protein-based blackcurrant concentrate encapsulates. These functional properties are of great importance for the further application in real food systems. The molecular docking study demonstrated the potential interaction manners of blackcurrant anthocyanins with whey protein molecules against α-amylase. High performance liquid chromatography confirmed the effective delivery of the blackcurrant anthocyanins by protein matrix via spray-drying and freeze-drying.

The whey protein-based blackcurrant concentrate encapsulates were high in protein contents, natural colourants, antioxidants with hypoglycaemic effects, and unique functional properties, indicating that these encapsulates may be used as additives in high carbohydrates food products to reduce sugar content. Thus, in Chapter 5, the cookie was chosen as a model product, and these encapsulates were added to a cookie formulation at different replacement levels (0, 5, 10, and 15%). The effects on the physicochemical and nutritional properties of the cookies were recorded. This addition not only improved the nutritional properties (high protein content, antioxidant activity) but also generated hypoglycaemic effects.

Chapter 7, 8, and 9 were focusing on the longitudinal study of sodium caseinate-based blackcurrant concentrate encapsulates. The physicochemical characteristics of sodium caseinate differed from those of whey protein isolate, such as solubility and molecular weight. Thus, a parallel investigation, including physicochemical properties, nutritional properties, functional properties and their practical application in a real food system (cookie), was conducted for the sodium caseinate-based encapsulates obtained via spray- and freeze-drying. Cookie products with different customer acceptance have been recognised.

10.2 Horizontal comparison

Whey protein isolate is a group of water-soluble globular protein molecules in a wide acidic pH range, providing a condition for the interactions with other water-soluble molecules. Sodium caseinate is completely denatured and without secondary and tertiary structure under physiological conditions. Its water solubility depends on the concentrate and pH conditions. Its amphipathicity resulted from it hydrophilic and hydrophobic regions of casein, which is likely to form micelles. These micelle structures provide protection for water-insoluble polyphenols. The main blackcurrant polyphenols are water-soluble.

In chapter 4, 10% (w/w) whey protein isolate aqueous solution was used to produce the encapsulates partly out of consideration for drying efficiency. The end point of titration was set up at pH = 4.5 due to the formation of the maximum amount of protein-polyphenol complexes at the protein isoelectric point. While in Chapter 7, 5% (w/w) sodium caseinate aqueous solution was used to produce the encapsulates partly out of consideration for its water solubility. The end point of titration was set up at pH = 6.0 due to the formation of flocs. As to the rest of the procedures producing the encapsulates, Chapter 4 and 7 followed the same procedures, resulting in the formation of encapsulates with unique properties.

In Chapter 5, cyanidin and delphinidin were detected in SWB at 265.20 ± 4.32 and 314.73 ± 3.80 μg/g, respectively, while in FWB, the content of cyanidin and the content of delphinidin were 183.91 ± 0.05 and 217.00 ± 0.02 μg/g, respectively. In Chapter 8, cyanidin and delphinidin were identified in SNaB, with respective value as 82.55 ± 1.04 and 71.39 ± 1.07 μg/g. However, in FNaB, the content of cyanidin and delphinidin was 59.55 ± 0.54 and 53.61 ± 0.13 μg/g, respectively. The anthocyanin's delivery efficiency of whey protein was three times higher than that of sodium caseinate. Meanwhile, spray-dried encapsulates of SWB and SNaB had higher anthocyanins content than freeze-dried encapsulates of FWB and FNaB), which may be attributed to the difference in releasing mechanisms and surface areas. The nutritional values, including antioxidant activity, total phenolic content, hypoglycaemic effects, and enzyme inhibitory activity were all related to the anthocyanins content, and all of these have been discussed in Chapter 4 and 7.

In Chapter 4 and 7, hypoglycaemic effects of the protein encapsulates have been demonstrated with less reducing sugar content after *in vitro* digestion. Molecular docking and enzymatic inhibitory study have been carried out to give further insight into the mechanisms of hypoglycaemic effects.

Functional properties, including water holding capacity, oil holding capacity, foamability, foam stability, and bulk density of protein ingredients is of critical importance for their practical application. In Chapter 5 and 8, functional properties of whey protein-based encapsulates and sodium caseinate were defined for the purpose of further practical application.

The novel protein ingredients had the potential to develop functional food products. In Chapter 6 and 9, cookie was chosen as a model food product. The sodium caseinate-based blackcurrant concentrate encapsulates were incorporated into the flour at various levels (0, 5, 10, and 15%) to reduce sugar content, increase protein content, and develop the cookies with different consumer acceptance. The protein sources and drying strategies of these novel protein ingredients have direct relationship with cookie physical properties. The anthocyanins content was positively related to the nutritional profiles of the cookies.

10.3 General conclusions

- Sustainability: broadening the practical utilisation of industrial by-products, including whey protein ingredients, and fruits and vegetable extracts or concentrates
- Human health: providing novel protein ingredients containing health benefit bioactive compounds
- Feasibility: spray-drying is a common encapsulation technique with low cost, high efficiency, and wide availability
- Practical applicability: application of the novel protein ingredients on starchy food matrix has the potential to replace sugar, increase protein

10.4 Future research

The industrial ingredient-whey protein isolate was chosen in this project as a wall material, which was manufactured by stirred-bed ion exchange adsorption process. pH of the proteins was adjusted, and the proteins are eluted from the ion exchanger, concentrated by

ultrafiltration and spray-dried, resulting in whey protein isolate with more than 90% protein content (Morr & Ha, 1993). The whey protein isolate powder was reconstituted before combined with blackcurrant concentrate under certain conditions. Afterwards, the protein solution was put to spray-drying process again, resulting in the excessive processing, energy loss, and protein denaturation.

However, if the first step of industrial spray-drying process, where the whey protein isolate protein solution was used as a carrier for bioactive compound, was not employed, the aforementioned issues (excessive processing, energy loss, and protein denaturation) might be avoid, and thus resulting in the formation novel protein ingredients with different properties. Another industrial ingredient, sodium caseinate, was used as a protein carrier for blackcurrant concentrate in this project. The addition of bioactive matrix, and the subsequent secondary drying process substantially changed the nutrition and the functionality of sodium caseinate. It is a promising research direction in the future that combining bioactive compounds with casein micelles, from a molecular level of complexation and/or conjugation, and a mixture level of combination, would benefit for the development of the cheese product and novel casein-based protein ingredients.

Toxicological data need to be collected at cellular level and mouse model in order to make sure the safe application of the material on food.

Whey protein has been recognised as a protein source for muscle growth and repair due to branched chain amino acids (valine, leucine, and isoleucine) (Kawaguchi, Izumi, Charlton, & Sata, 2011). Blackcurrant has been reported to improve cardiovascular function by supporting blood flow, reducing muscle fatigue, and the onset of sourness during and post exercise (Bowtell & Kelly, 2019). Thus, their combined products have the potential to benefit for sports performance which need to be further studied.

The customer acceptance of these encapsulates-enriched cookies should be evaluated. Application of the novel protein ingredients in more real food systems is necessary to broaden their practical application.

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